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(71) Applicant *(for all designated States except US)*: **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **HUANG, Xudong** [US/US]; Andover, MA (US).

(74) Agent: **JARRELL, Brenda, Herschbach**; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).

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(54) Title: BIFUNCTIONAL MOLECULES COMPRISING AT LEAST ONE INTEGRIN-BINDING AND THEIR USE IN IMAGING AND THERAPY OF ANGIOGENESIS AND RELATED DISORDERS

(57) Abstract: The present invention relates to the diagnosis, prevention, and treatment of pathophysiological conditions associated with angiogenesis. Bifunctional therapeutic molecules, contrast imaging agents and boron-containing compounds exhibiting a high affinity and specificity for integrins, and pharmaceutical compositions thereof are described. The invention also provides methods of using these bifunctional molecules, contrast imaging agents, and boron-containing compounds for imaging angiogenesis *in vitro* and *in vivo*; and for preventing or treating angiogenic disorders associated with an up-regulation of the angiogenic process leading to excessive blood vessel prolifération, such as cancer, psoriasis, rheumatoid arthritis, atherosclerosis, restenosis, and ocular neovascularization.

BIFUNCTIONAL MOLECULES COMPRISING AT LEAST ONE INTEGRIN-BINDING MOIETY AND THEIR USE IN IMAGING AND THERAPY OF ANGIOGENESIS AND RELATED DISORDERS

Government Interests

[0001] The work described herein was funded by the National Institutes of Health/National Institute of Mental Health (Grant No. 5K01 MH002001-02). The United States government may have certain rights in the invention.

Related Application

[0002] This application claims priority to Provisional Patent Application No. 60/443,832, filed January 30, 2003, which is incorporated herein by reference in its entirety.

Background of the Invention

[0003] The development of new blood vessels from pre-existing vasculature is a process known as angiogenesis. New blood vessels are generated by a complex interaction between endothelial cells, basement membrane, and extracellular matrix (ECM) (T. Korff *et al.*, J. Cell. Sci. 1999, 112: 3249-3258). In the angiogenic cascade of events leading to vessels growth, an appropriate stimuli produced locally induces proliferation and migration of endothelial cells (A. Yoshida *et al.*, Growth Factors, 1996, 13: 57-64). This is accompanied by an extensive local remodeling resulting in the formation of new blood vessels, which acquire a supporting framework from the surrounding ECM (P. Wesseling *et al.*, J. Neuro-Oncol. 1997, 32: 253-265). Angiogenesis is involved in normal physiological processes such as embryogenesis, the female reproductive cycle, wound healing, and regeneration of deciduous tissues (P.A. D'Amore and R.W. Thompson, Annu. Rev. Physiol. 1987, 49: 453-464; J. Folkman and Y. Shing, J. Biol. Chem. 1992, 267: 10931-10934). In adults, angiogenesis occurs only locally and transiently, and is regulated by a tightly controlled system of angiogenic stimulators and inhibitors.

[0004] However, under certain pathological circumstances, the body loses its control of angiogenesis, and an imbalance between angiogenic stimulators and inhibitors causes either excessive or insufficient blood vessel growth. Altered regulation of the angiogenic process is now recognized to be responsible for a wide

range of human disorders (C.H. Blood and B.R. Zetter, Biochem. Biophys. Acta, 1990, 1032: 89-118; J. Folkman, Semin. Cancer Biol. 1992, 3: 65-71; D. Weinstat-Saslow and P.S. Steeg, FASEB J. 1994, 8: 401-407). For example, ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing. By contrast, excessive blood vessel proliferation may favor the development and progression of clinical conditions such as cancer, psoriasis, atherosclerosis, restenosis, a number of inflammatory disorders (*e.g.*, rheumatoid arthritis), and ocular neovascularization.

[0005] Progress in the understanding of the molecular basis of angiogenic regulation led to the identification of key endogenous controlling factors (J. Folkman and P.A. D'Amore, Cell, 1996, 87: 1153-1155; T. Browder *et al.*, J. Biol. Chem. 2000, 275: 1521-1524; and P. Carmeliet, Nat. Med. 2000, 6: 389-395). Identified stimulators include, among others, matrix metalloproteases, angiopoietins, integrins, and cadherins; inhibitors include thrombospondins, endostatin, angiostatin, troponin, and interleukins, to name a few. The complexity of the angiogenic process, which involves both positive and negative regulators, provides a number of targets for therapy. Overall, there are currently more than 25 different anti-angiogenic agents (for an updated list, see: http://www.cancer.gov/clinicaltrials/developments/anti-angio-table) in phase I through phase III clinical trials.

[0006] In particular, pharmaceutical companies are actively developing therapeutics that target integrins. Integrins are a large family of cell surface receptors that bind a wide variety of ECM ligands (R.O. Hynes, Cell, 1992, 69: 11-25) and mediate numerous cell-cell and cell-matrix interactions. Alterations or aberrations in cell-adhesion, and in particular integrin-mediated cell-adhesion, have been implicated in the pathogenesis of a number of both acute and chronic diseases, making integrins attractive targets for the development of therapeutic agents. Various integrin-antagonist candidates, including antibodies, cyclic peptides, peptidomimetics, and small molecules have so far been identified (G.P. Curley *et al.*, Cell Mol. Life Sci. 1999, 56: 427-441).

[0007] One of the most promising therapeutic targets for cancer therapy is the integrin $\alpha_v\beta_3$, which has been reported to affect tumor growth, local invasiveness, and metastatic potential (P. Clezardin, Cell Mol. Life Sci. 1998, 54: 541-548). Similar to

other integrins, $\alpha_v\beta_3$ recognizes ECM ligands with an exposed arginine-glycine-aspartic acid (or RGD) sequence and mediates the adhesion and migration of different types of tumor cells (S.M. Albelda *et al.*, Cancer Res. 1990, 5: 6757-6764; C.L. Gladson and D.A. Cheresh, J. Clin. Invest. 1991, 88: 1924-1932; R.M. Lafrenie *et al.*, Eur. J. Cancer, 1994, 30: 2151-2158). Furthermore, $\alpha_v\beta_3$, which is only weakly expressed on resting endothelial cells, is highly up-regulated on activated endothelial cells and plays an important role in the angiogenic process. Indeed, antibodies and peptides that block $\alpha_v\beta_3$ have been shown to cause rapid tumor cell apoptosis (P.C. Brooks *et al.*, Science, 1994, 264: 569-571) and to inhibit tumor growth in animal models (P.C. Brooks *et al.*, Cell, 1994, 79: 1157-1164; C.P. Carron *et al.*, Cancer Res. 1998, 58: 1930-1935; J.S. Kerr *et al.*, Anticancer Res. 1999, 19: 959-968; H.N. Lode *et al.*, Proc. Natl. Acad. Sci. USA, 1999, 96: 1591-1596).

[0008] Recently, a novel anti-angiogenic strategy involving the modulation of total body copper status has been proposed as a treatment for cancer. It has long been known that copper metabolism is greatly altered in human cancer patients and tumor-bearing animals, and that serum copper levels correlate with tumor incidence, burden, progression, and recurrence in a variety of human cancers. Furthermore, preclinical and *in vitro* studies have demonstrated that copper is an important factor stimulating tumor angiogenesis (M. Ziche *et al.*, J. Natl. Cancer Inst. 1982, 69: 475-482; K.S. Raju *et al.*, J. Natl. Cancer Inst. 1982, 69: 1183-1188). The first human trial of an anticopper approach showed that the use of tetrathiomolybdate as metal chelator to deprive cancer tumors of the copper supply they need to form new blood vessels stopped the progression of the disease and prevented metastasis in a small group of advanced patients for more than a year (G.J. Brewer *et al.*, Clin. Cancer Res. 2000, 6: 1-10). Compared with therapeutic strategies using antagonists of specific key regulators, this anticopper approach has the advantage of affecting multiple angiogenic stimulators and, consequently, is more generally applicable. However, most metal-chelators are non-specific and may perturb the normal physiological function(s) of other metal-requiring biomolecules, thus creating potential side-effects that may prove too great for clinical use.

[0009] Although novel promising angiogenesis inhibitors are constantly being developed, a more controlled and systematic design of therapeutic agents is impeded by lack of adequate methods for monitoring treatment and drug effects. Anti-tumor

activity is currently assessed by determination of the reduction in tumor size, a method that cannot be applied when the therapeutic goal is to stabilize an existing condition or to prevent metastasis. A number of imaging techniques, in particular functional Magnetic Resonance Imaging (MRI) and nuclear techniques, have been used for studying microvascular circulation in tumors (R. Brasch *et al.*, J. Magn. Reson. Imaging, 1997, 7: 68-74; J. Dennie *et al.*, Magn. Reson. Med. 1998, 40: 793-799; R. Weissleder *et al.*, Eur. J. Cancer, 1998, 34: 1448-1454; J.S. Taylor *et al.*, J. Magn. Reson. Imaging, 1999, 10: 903-907). Pioneering imaging studies have targeted specific endothelial markers (D. Neri *et al.*, Nat. Biotechnol. 1997, 15: 1271-1275; V. Brower, Nat. Biotechnol. 1999, 17: 963-968; and S. Bredow e*t al.*, Eur. J. Cancer, 2000, 36: 675-681). Examples of $\alpha_v\beta_3$ targeting agents include antibody-coated paramagnetic liposomes (D.A. Sipkins *et al.*, Nat. Med. 1998, 5: 623-626); radiolabeled linear decapeptides (G.B. Sivolapenko *et al.*, Eur. J. Nucl. Med. 1998, 25: 1383-1389); and radiolabeled RGD derivatives (R. Haubner *et al.*, J. Nucl. Med. 1999, 40: 1061-1071). Although the results obtained in these studies are encouraging and attest of the feasibility of the approach, unfavorable pharmacokinetics, low tumor uptake, and weak tumor-to-organ ratios have also been reported for these targeted imaging agents, suggesting that more work is necessary before they can be used clinically.

[0010] Therefore, improved methods and compositions are still needed for the control and inhibition of abnormal angiogenesis, and new systems are highly desirable for the imaging of angiogenesis, both for diagnostic purposes and for drug development, treatment planning and monitoring.

Summary of the Invention

[0011] The present invention relates to the diagnosis, prevention, and treatment of pathophysiological conditions associated with angiogenesis. In particular, the invention encompasses systems for imaging angiogenesis in patients as well as reagents and strategies for preventing and treating diseases and disorders associated with an up-regulation of the angiogenic process leading to excessive blood vessel proliferation. In certain preferred embodiments, the invention allows identification, production, and/or use of agents that act as antagonists of integrins by inhibiting (*e.g.*,

precluding, reversing or disrupting) the binding of integrins to their endogenous protein ligands.

[0012] In one aspect, the invention provides therapeutic reagents that can have a dual mode of action by acting as metal chelators and as integrin-antagonists. More specifically, the present invention provides bifunctional molecules comprising at least one integrin-inhibiting moiety associated with at least one metal-chelating moiety. In certain preferred embodiments, the integrin-inhibiting moiety inhibits the biological activity of integrins, preferably of α_v integrins, more preferably of the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; most preferably of the integrin $\alpha_v\beta_3$. In other preferred embodiments, the integrin-inhibiting moiety inhibits the biological activity of integrins by precluding, reversing, or otherwise disrupting the binding of integrins to their endogenous protein ligands. Preferably, the integrin-inhibiting moiety has a high binding affinity and specificity for the α_v integrins. More preferably, the integrin-inhibiting moiety has a high binding affinity and specificity for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins. Most preferably, the integrin-inhibiting moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$. In other preferred embodiments, the metal-chelating moiety binds transition metal ions that are relevant to the angiogenic process, such as copper II (Cu^{2+}) and iron III (Fe^{3+}). Preferably, the metal-chelating moiety binds with high affinity and specificity copper II (Cu^{2+}).

[0013] In another aspect, the invention provides targeted reagents that act as integrin-antagonists and are detectable by imaging techniques. More specifically, the invention provides contrast imaging agents comprising at least one imaging moiety associated with at least one integrin-binding moiety. In certain preferred embodiments, the integrin-binding moiety acts as an integrin-antagonist by binding to the integrin, thereby preventing its interaction with endogenous protein ligands. Preferably, the integrin-binding moiety has a high binding affinity and specificity for α_v integrins; more preferably for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; most preferably for the integrin $\alpha_v\beta_3$. The imaging moiety may be any suitable entity that is detectable by imaging techniques. In certain preferred embodiments, the imaging moiety comprises at least one metal-chelating moiety complexed to a detectable metal entity. Preferably, the metal-chelating moiety is complexed to a physiologically acceptable metal entity. In other preferred embodiments, the metal entity is a paramagnetic

metal ion and the contrast imaging agent is detectable by Magnetic Resonance Imaging (MRI). Preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}). In yet other preferred embodiments, the metal entity is a radionuclide and the contrast imaging agent is detectable by Single Photon Emission Computed Tomography (SPECT). Preferably, the radionuclide is technetium-99m (^{99m}Tc).

[0014] The invention also provides contrast imaging agents comprising at least one metal-chelating moiety associated with at least one integrin-binding moiety labeled with a magnetically active nuclei detectable by Magnetic Resonance Spectroscopy (MRS). Preferably, the magnetically active nuclei is natural isotope carbon-13 (^{13}C) or fluorine-19 (^{19}F).

[0015] In another aspect, the invention provides targeted molecules that show some degree of attraction for integrins and can be used as therapeutics in Boron Neutron Capture Therapy. More specifically, the present invention provides compounds comprising at least one integrin-binding moiety associated with a boron-containing moiety. In certain preferred embodiments, the integrin-binding moiety has a high binding affinity and specificity for integrins. Preferably, the integrin-binding moiety has a high binding affinity and specificity for α_v integrins, more preferably for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; most preferably for the integrin $\alpha_v\beta_3$. The invention also provides boron-containing compounds comprising at least one metal-chelating moiety associated with at least one integrin-binding moiety labeled with boron-10. Preferably, the metal-chelating moiety binds with high affinity and specificity copper II. The invention further provides boron-containing compounds comprising at least one imaging moiety associated with at least one integrin-binding moiety labeled with boron-10. In certain preferred embodiments, the imaging moiety comprises at least one metal-chelating moiety complexed to a paramagnetic metal ion that is detectable by MRI. Preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}). In other preferred embodiments, the imaging moiety comprises at least one metal-chelating moiety complexed to a radionuclide that is detectable by SPECT. Preferably, the radionuclide is technetium-99m (^{99m}Tc).

[0016] In yet another aspect, the invention provides pharmaceutical compositions. The inventive pharmaceutical compositions comprise at least one reagent of the invention, or a physiologically tolerable salt thereof, and at least one pharmaceutically

acceptable carrier. In these pharmaceutical compositions, the reagent is present in an amount sufficient to fulfill its intended purpose. More specifically, the present invention provides pharmaceutical compositions comprising an effective amount of at least one bifunctional therapeutic molecule, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier. Also provided are pharmaceutical compositions comprising an imaging effective amount of at least one contrast imaging agent, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier. Also provided are pharmaceutical compositions comprising a BNCT effective amount of at least one boron-containing compound, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier.

[0017] In still another aspect, the invention provides methods for preventing or inhibiting angiogenesis *in vitro* and *in vivo*. In certain preferred embodiments, the present invention allows prevention or inhibition of angiogenesis by inhibiting the biological activity of integrins. Preferably, the inventive methods prevent or inhibit angiogenesis by inhibiting the biological activity of α_v integrins; more preferably of the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; most preferably, of the $\alpha_v\beta_3$ integrin. In other preferred embodiments, the present invention allows the prevention or inhibition of angiogenesis by inhibiting the biological action of angiogenic stimulators that require the presence of copper II as co-factor. Preferably, the inventive methods prevent or inhibit angiogenesis by precluding, reversing, disrupting or otherwise interfering with the interaction of these angiogenic stimulators with copper II.

[0018] More specifically, methods are provided for preventing or inhibiting angiogenesis in a system, comprising contacting the system with a bifunctional molecule of the invention, or a pharmaceutical composition thereof. The system may be any biological entity known in the art to be able to undergo any of the steps of the angiogenic process, such as proliferation and/or migration of endothelial cells, formation of new blood vessels, increased vascularity of an organ or tissue of the body, and metastasis. The system may be a cell, a biological fluid, a biological tissue, or an animal. When the system is a cell, a biological fluid, or a biological tissue, it may, for example, originate from a live patient (*e.g.*, it may be obtained by biopsy) or a deceased patient (*e.g.*, it may be obtained at autopsy). The patient may be a human or another mammal. In certain preferred embodiments, the cell, biological fluid, or

biological tissue originates from a patient suspected of having a pathophysiological condition associated with angiogenesis.

[0019] Also provided herein are methods for treating a patient with a clinical condition associated with angiogenesis, comprising administering to the patient an effective amount of a bifunctional molecule of the invention, or a pharmaceutical composition thereof. In certain preferred embodiments, the clinical condition affecting the patient is selected from the group consisting of cancer, psoriasis, atherosclerosis, restenosis, rheumatoid arthritis, and ocular neovascularization. In other preferred embodiments, the inventive method is used to prevent the formation of metastases in a cancer patient.

[0020] In yet another aspect, the invention provides methods for imaging angiogenesis *in vitro* and *in vivo*. In preferred embodiments, the inventive methods involve the use of targeted contrast imaging agents and imaging techniques.

[0021] More specifically, the invention provides methods for imaging angiogenesis in a system comprising contacting the system with an imaging effective amount of a contrast imaging agent, or a pharmaceutical composition thereof. The contacting is carried out under conditions that allow the contrast imaging agent to interact with and bind to integrins. The presence of integrins bound to the contrast imaging agent is then detected using an imaging technique, and one or more images of at least part of the system are generated. In certain preferred embodiments, the inventive method is used for identifying potential therapeutic agents for the treatment of angiogenic disorders. The invention includes the therapeutic agents identified by this method.

[0022] The present invention also provides methods for imaging angiogenesis in a patient. These methods comprise administering to the patient an imaging effective amount of a targeted contrast imaging agent, or a pharmaceutical composition thereof. The administration is carried out under conditions that allow the contrast imaging agent to reach the part(s) of the body affected with angiogenesis, and to interact with and bind to integrins. The presence of integrins bound to the contrast imaging agent is then detected using an imaging technique, and one or more images of at least part of the patient's body are generated.

[0023] In certain preferred embodiments, the inventive methods for imaging angiogenesis in a system or a patient are carried out by using a contrast imaging agent, wherein the imaging moiety comprises at least one metal-chelating moiety complexed to a paramagnetic metal ion; the detection is performed by Magnetic Resonance Imaging (MRI); and magnetic resonance images are generated. Preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}). In other preferred embodiments, the inventive methods are carried out by using a contrast imaging agent, wherein the imaging moiety comprises at least one metal-chelating moiety complexed to a radionuclide; the detection is performed by Single Photon Emission Computed Tomography (SPECT); and SPECT images are generated. Preferably, the radionuclide is technetium-99m (^{99m}Tc). In still other preferred embodiments, the inventive methods are carried out by using a contrast imaging agent, wherein the integrin-binding moiety is labeled with a magnetically active nuclei; the detection is performed by Magnetic Resonance Spectroscopy (MRS); and MR images are generated. Preferably, the magnetically active nuclei is the natural isotope carbon-13 (^{13}C) or fluorine-19 (^{19}F).

[0024] In certain preferred embodiments, the inventive methods are used to detect the presence of and/or localize angiogenesis in a patient (*i.e.*, to detect the presence of and/or localize proliferating endothelial cells, newly formed or growing blood vessels, increased vascularity of an organ or a tissue of the body, and/or metastases). In other preferred embodiments, the inventive methods are used to diagnose an angiogenic disorder. For example, the methods may be used to diagnose a clinical condition selected from the group consisting of cancer, psoriasis, atherosclerosis, restenosis, rheumatoid arthritis, and ocular neovascularization. In other preferred embodiments, the methods are used to identify, characterize and classify a type of tumor. In still other preferred embodiments, the methods are used to follow the progression of a pathophysiological condition associated with angiogenesis. In yet other preferred embodiments, the methods are used to monitor the response of a patient to a treatment for a pathophysiological condition associated with angiogenesis.

[0025] In another aspect, the present invention provides methods for treating a patient with a malignant tumor comprising steps of administering to the patient a BNCT effective amount of a boron-containing compound, or a pharmaceutical composition thereof, under conditions that allow the boron-containing compound to

(1) reach the part of the patient's body where the tumor is located and (2) concentrate in the tumor by binding to integrins; and exposing said tumor and said compound to a neutron radiation beam of an energy such that said compound emits α particles in an amount sufficient to shrink said tumor. The inventive methods may be used to treat patients with brain tumors, such as malignant glioma of the brain and gliobastoma, or with malignant melanoma.

[0026] The bifunctional therapeutic molecules, targeted contrast imaging agents, boron-containing compounds, pharmaceutical compositions, and methods described herein can also be used to diagnose, prevent or treat angiogenesis-related conditions affecting mammals other than humans. Other aspects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only.

Definitions

[0027] Throughout the specification, several terms are employed, that are defined in the following paragraphs.

[0028] The term "***angiogenesis***", as used herein, refers to a multi-step process which results in the formation of new blood vessels and/or in an increase in the vascularity of an organ or tissue of the body. Under normal physiological conditions, angiogenesis is highly regulated by a system of angiogenic stimulators and inhibitors, and occurs only in very specific situations, including wound healing, embryonal and fetal development, and formation of the corpus luteum, endometrium and placenta. In certain disease states, the control of angiogenesis is altered and an imbalance between angiogenic stimulators and inhibitors induces an up-regulation or a down-regulation of the angiogenic process. In cancer, "angiogenesis" refers to the process by which tumor cells trigger abnormal blood vessel growth to create their own blood supply and get needed nutrients to grow and metastasize in other locations in the body.

[0029] The terms "***angiogenic disorders***", "***angiogenic diseases***", and "***pathophysiological conditions associated with angiogenesis***" are used herein interchangeably. In the context of the present invention, they refer to clinical

conditions involving up-regulation of the angiogenic process leading to excessive blood vessel formation. These conditions include, but are not limited to, cancer, psoriasis, atherosclerosis, restenosis, a number of inflammatory disorders (*e.g.*, rheumatoid arthritis), and ocular neovascularization (leading to diabetic retinopathy, neovascular glaucoma, age-related macular degeneration, or retinal vein occlusion).

[0030] The term "***cancer***", as used herein, refers to malignant tumors or carcinoma. The term "***tumor***" is used herein to describe an abnormal tissue growth, which occurs when the proliferation of tumor cells is more rapid than that of normal cells and continues even after the stimuli that initiated the new growth has ceased. Tumors generally exhibit partial or complete lack of structural organization and functional coordination with the normal tissue, and usually form a distinct mass of tissue, which may be benign (benign tumor) or malignant (carcinoma). Malignant tumors may invade surrounding tissues and undergo metastasis, and are likely to recur and to cause death of the patient unless adequately treated.

[0031] The term "***anti-angiogenic activity***", as used herein, refers to the capability of a molecule, compound, agent, or treatment to prevent or inhibit angiogenesis. For example, an "***anti-angiogenic agent***" is capable of preventing, inhibiting, or otherwise interfering with one or more steps of the angiogenic process leading to excessive blood vessel proliferation.

[0032] The term "***integrin***", as used herein, refers to any of the many cell surface receptor proteins, also known as adhesion protein receptors, which bind extracellular matrix ligands or other cell adhesion protein ligands and thereby mediate cell-cell and cell-matrix adhesion processes. Integrins constitute a superfamily of membrane receptors that are encoded by genes belonging to a gene superfamily and are typically composed of heterodimeric transmembrane glycoproteins containing an α- and a β-subunit. Members of an integrin subfamily have a common β subunit, which can combine with different α subunits to form adhesion protein receptors with different specificities. Among the known α subunits, the α_v subunit seems to be the most promiscuous, forming heterodimers with six different β subunits. Integrins α_v include, for example, the receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

[0033] The terms "***integrin-antagonist***" and "***integrin-inhibitor***" are used herein interchangeably. They refer to molecules, agents or compounds that are capable of inhibiting the biological activity of integrins.

[0034] The term "***integrin-inhibiting moiety***" refers to any entity capable of inhibiting the biological activity of integrins. An integrin-inhibiting moiety may alter cell-matrix and cell-cell adhesion processes, by inducing alterations in the integrin conformation and/or in the integrin cytoskeleton, or by causing disassociation of the α and β subunits (or any of the parts thereof), or disassociation of integrin clusters and/or of clusters formed between integrins and other proteins. An integrin-inhibiting moiety may also act directly on the receptor by blocking binding to the integrin extracellular region, for example, by binding to a portion of at least one subunit (α or β) of the integrin.

[0035] An "***integrin-binding moiety***", as defined herein, is an integrin-inhibiting moiety that specifically acts by binding to integrins, thereby precluding, reversing, inhibiting or otherwise interfering with the binding of integrins to their endogenous ligands. Preferably, integrin-binding moieties exhibit a high binding affinity and specificity for α_v integrins; more preferably, for the integrins $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$; most preferably, for the $\alpha_v\beta_3$ integrin. When an integrin-binding moiety is part of a molecule, it confers its property to the molecule, and the molecule becomes "***targeted***" to integrins (*i.e.*, it specifically and efficiently binds to integrins). The binding between integrins and an integrin-binding moiety may be covalent or non-covalent (*e.g.*, hydrophobic interactions, electrostatic interactions, dipole interactions, van der Waals interactions, hydrogen bonding, *etc.*). Most often the binding is non-covalent.

[0036] The terms "***binding affinity***" and "***affinity***" are used herein interchangeably and refer to the level of attraction between molecular entities. Affinity can be expressed quantitatively as a dissociation constant (K_d), or its inverse, the association constant (K_a). In the context of this invention, two types of affinity are considered: (1) the affinity of an integrin-binding moiety for integrins, and (2) the affinity of a metal-chelating moiety for a transition metal ion, or for another metal entity.

[0037] The terms "***metal-chelating***" and "***chelating***", as applied herein to chemical moieties, agents, compounds, or molecules refer to the ability of an entity characterized by the presence of two or more polar groups to participate in the formation of a complex (containing more than one coordinate bond) with a transition metal ion or another metal entity. Metal-chelating agents are known in the art.

[0038] In the context of the present invention, the term "***bifunctional molecule***" refers to a molecule which comprises at least one metal-chelating moiety associated with at least one integrin-inhibiting moiety, and which, consequently, has a dual mode of action. More specifically, bifunctional molecules of the invention can act both as metal chelators and as integrin-antagonists.

[0039] As used herein, the term "***transition metal ion***" refers to ionic forms of elements known in the art as transition metals, more particularly to copper II (Cu^{2+}) and iron III (Fe^{3+}).

[0040] The term "***contrast imaging agent***", as used herein, refers to any entity that can be used to detect specific biological elements using imaging techniques. More specifically, contrast imaging agents of the invention can be used to image angiogenesis in *in vitro*, *in vivo*, and *ex vivo* systems as well as in patients. Contrast imaging agents of the invention are targeted molecules comprising at least one imaging moiety associated with at least one integrin-binding moiety. In preferred embodiments of the invention, the imaging moiety in the contrast imaging agent comprises at least one metal-chelating agent complexed to a detectable metal entity. Other contrast imaging agents of the invention comprise at least one metal-chelating moiety associated with at least one integrin-binding moiety labeled with a magnetically active nuclei. Boron-containing compounds that comprise at least one imaging moiety associated with at least one integrin-binding moiety labeled with boron-10, are also contrast imaging agents of the invention.

[0041] As used herein, the term "***metal entity***" refers to a paramagnetic metal ion that is detectable by imaging techniques such as Magnetic Resonance Imaging (MRI), or to a radionuclide, that is detectable by imaging techniques such as Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET).

[0042] A used herein, the term "***paramagnetic metal ion***" refers to a physiologically tolerable entity that can be complexed by a metal-chelating agent and is detectable by MRI. Preferably, the paramagnetic metal ion is selected from the group consisting of gadolinium III (Gd^{3+}), chromium III (Cr^{3+}), dysprosium III (Dy^{3+}), iron III (Fe^{3+}), manganese II (Mn^{2+}), and ytterbium III (Yb^{3+}).

[0043] As used herein, the term "***radionuclide***" refers to a radioactive isotope of a metallic element that can be complexed by a metal-chelating agent and used in radiopharmaceutical techniques. Preferred radionuclides are technetium-99m (^{99m}Tc), gallium-67 (^{67}Ga), yttrium-91 (^{91}Y), indium-111 (^{111}In), rhenium-186 (^{186}Re), and thallium-201 (^{201}Tl).

[0044] As used herein, the term "***magnetically active nuclei***" refers to a nuclei that is detectable by nuclear Magnetic Resonance Spectroscopy (MRS). Preferred magnetically active nuclei for use in the present invention are the natural isotopes carbon-13 (^{13}C) and fluorine-19 (^{19}F).

[0045] In the context of the present invention, the term "***targeted boron-containing compound***" refers to a molecule, compound, or agent that shows some degree of attraction for integrins and can be used as therapeutic agent in Boron Neutron Capture Therapy (BNCT).

[0046] The terms "***Boron Neutron Capture Therapy***" and "***BNCT***" are used herein interchangeably. They refer to an investigational form of a two-part radiation therapy, which has the potential ability to selectively kill tumor cells without affecting surrounding healthy tissues. BNCT is based on a capture reaction between Boron-10 and neutrons, which generates α particles that can destroy tumor cells.

[0047] The term "***prevention***" is used herein to characterize a method that is aimed at delaying or preventing the onset of a pathophysiological condition associated with angiogenesis. The term "***treatment***" is used herein to characterize a method that is aimed at (1) delaying or preventing the onset of an angiogenic disorder; or (2) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disorder; or (3) bringing about ameliorations of the symptoms of the disorder; or (4) curing the disorder. The treatment may be administered prior to the onset of the disease, for a prophylactic or preventive action. It may also be administered after initiation of the disease, for a therapeutic action.

[0048] The terms "***individual***" and "***patient***" are used herein interchangeably. They refer to a human or another mammal, that can be affected by a pathophysiological condition associated with angiogenesis but may or may not have an angiogenic disease.

[0049] As used herein, the term "***system***" refers to a biological entity that is known in the art to be able to undergo angiogenesis. In the context of this invention, *in vitro*, *in vivo*, and *ex vivo* systems are considered; and the system may be a cell, a biological fluid, a biological tissue, or an animal. A system may, for example, originate from a live patient (*e.g.*, it may be obtained by biopsy), or from a deceased patient (*e.g.*, it may be obtained at autopsy). The patient may be a human or another mammal.

[0050] As used herein, the term "***biological fluid***" refers to a fluid produced by and obtained from a patient. Examples of biological fluids include, but are not limited to, cerebrospinal fluid (CSF), blood serum, urine, and plasma. In the present invention, biological fluids include whole or any fraction of such fluids derived by purification, for example, by ultrafiltration or chromatography.

[0051] As used herein, the term "***biological tissue***" refers to a tissue obtained from a patient. The biological tissue may be whole or part of any organ or system in the body (*e.g.*, brain, pancreas, heart, kidney, gastrointestinal tract, thyroid gland, nervous system, eye, skin, and the like).

[0052] As used herein, the term "***effective amount***" refers to any amount of a therapeutic molecule of the invention, or pharmaceutical composition thereof, that is sufficient to fulfill its intended purpose(s) (*e.g.*, the purpose(s) may be: to delay or prevent the onset of a pathological condition associated with angiogenesis; to slow down or stop the progression, aggravation, or deterioration of the symptoms of the condition (for example, to prevent the formation of metastases in a cancer patient); to bring about ameliorations of the symptoms of the condition; or to cure the condition). More specifically, in the case of a bifunctional therapeutic molecule, the purpose(s) may be: to prevent, inhibit, reverse, or otherwise interfere with one or more steps of the angiogenic process that lead to proliferation and/or migration of endothelial cells, growth of new blood vessels, increase in the vascularity of an organ or tissue of the body, and/or to metastasis. This can, for example, be achieved by inhibiting the

biological activity of integrins and/or by inhibiting the biological activity of angiogenic stimulators that require the presence of copper II as co-factor.

[0053] As used herein, the term "***imaging effective amount***" refers to any amount of a contrast imaging agent of the invention, or pharmaceutical composition thereof, that is sufficient to allow the imaging of angiogenesis in a system or a patient (*e.g.*, to allow the detection of the presence of proliferating and/or migrating endothelial cells, of newly formed or growing blood vessels, increased vascularity of an organ or tissue of the body, and/or of metastases).

[0054] As used herein, the term "***BNCT effective amount***" refers to any amount of a boron-containing compound of the invention, or pharmaceutical composition thereof, that is sufficient to accumulate selectively in a tumor and emit α particles to substantially shrink the tumor when the boron-containing compound and the tumor are exposed to a neutron radiation beam of adequate energy.

[0055] A "***pharmaceutical composition***" as used herein is defined as comprising at least one reagent of the invention (bifunctional therapeutic molecule, targeted contrast imaging agent, or boron-containing compound), or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier.

[0056] The term "***physiologically tolerable salt***" refers to any acid addition or base addition salt that retains the biological activity and properties of the free base or free acid, respectively, and that is not biologically or otherwise undesirable. Acid addition salts are formed with inorganic acids (*e.g.*, hydrochloric, hydrobromic, sulfuric, nitric, phosphoric acids, and the like); and organic acids (*e.g.*, acetic, propionic, pyruvic, maleic, malonic, succinic, fumaric, tartaric, citric, benzoic, mandelic, methanesulfonic, ethanesulfonic, *p*-toluenesulfonic, salicylic acids, and the like). Base addition salts can be formed with inorganic bases (*e.g.*, sodium, potassium, lithium, ammonium, calcium, magnesium, zinc, aluminum salts, and the like) and organic bases (*e.g.*, salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-dimethyl-aminoethanol, 2-diethylaminoethanol, trimethamine, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine,

methylglucamine, theobromine, purines, piperazine, piperidine, N-ethylpiperidine, polyamine resins, and the like).

[0057] As used herein, the term "***pharmaceutically acceptable carrier***" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not excessively toxic to the hosts at the concentrations at which it is administered. The term includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents, absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art (see, for example, Remington's Pharmaceutical Sciences, E.W. Martin, 18th Ed., 1990, Mack Publishing Co., Easton, PA).

[0058] Additional definitions are provided throughout the Detailed Description.

Detailed Description of Certain Preferred Embodiments

[0059] The present invention is directed to the diagnosis, prevention, and treatment of angiogenic disorders. More specifically, the present invention is directed to the diagnosis, prevention, and treatment of pathophysiological conditions associated with an up-regulation of the angiogenic process leading to excessive blood vessel proliferation. In particular, the invention encompasses systems for imaging angiogenesis *in vitro* and *in vivo* as well as reagents and strategies for preventing and treating angiogenic diseases including, for example, cancer, psoriasis, atherosclerosis, restenosis, a number of inflammatory disorders (*e.g.*, rheumatoid arthritis), and ocular neovascularization. In certain preferred embodiments, the invention allows identification, production, and/or use of agents that act as integrin-antagonists by inhibiting *(e.g.*, precluding, reversing, or disrupting) the binding of integrins to their endogenous ligands.

I. Bifunctional Therapeutic Molecules

[0060] One aspect of the present invention relates to a new class of therapeutic reagents with a dual mode of action.

[0061] Alterations and/or aberrations in integrin-mediated cell-adhesion have been implicated in a large number of both acute and chronic diseases, making

integrins attractive targets for the development of therapeutic agents. Indeed, the pharmaceutical industry has focused most of its efforts on the identification and production of integrin-antagonist candidates, including antibodies, cyclic peptides, peptidomimetics, and small molecules (G.P. Curley *et al.*, Cell Mol. Life Sci. 1999, 56: 427-441). Although it is well recognized that such anti-angiogenic agents, either singly or in combination with other therapeutics, will be in the mainstream of cancer therapy in the years ahead (N.J. Nelson, J. Natl. Cancer Inst. 1998, 90: 960-963; W.J. Gradishar, Invest. New Drugs, 1997, 15: 49-59), integrin-antagonists have the inherent disadvantage of being highly specific and therefore only applicable in a limited number of cases. On the contrary, the anticopper strategy to cancer treatment (G.J. Brewer *et al.*, Clin. Cancer Res. 2000, 6: 1-10) is more widely applicable as it can affect multiple angiogenic stimulators, whose biological activity depends on the presence of copper II as co-factor. However, the lack of specificity of most metal-chelators makes their clinical use improbable as they may perturb the normal physiological functions of other metal-requiring biomolecules, thereby creating potentially serious side-effects.

[0062] The present invention encompasses the hypothesis that (1) a metal-chelator targeted to integrins should have less undesirable side-effects than non-specific metal chelating agents, and (2) an integrin-antagonist with the ability of inhibiting the biological activity of other angiogenic stimulators should be more effective than the highly specific therapeutic agents currently developed. Accordingly, the present invention provides anti-angiogenic agents that are designed to have a dual mode of action, and can both inhibit the biological activity of integrins and act as metal-chelators. More specifically, the present invention provides bifunctional molecules comprising at least one metal-chelating moiety associated with at least one integrin-inhibiting moiety.

Integrin-inhibiting Moieties

[0063] An integrin-inhibiting moiety is any entity that is capable of inhibiting the biological activity of integrins. Preferably, integrin-inhibiting moieties are stable, non-toxic entities that retain their properties under *in vitro* and *in vivo* conditions. Other preferred properties of the integrin-inhibiting moieties are sustained biological effect (to reduce the dosing frequency), and targeting of angiogenic-specific

molecular pathways (to minimize the risk of toxicity). In certain preferred embodiments, the integrin-inhibiting moiety inhibits the biological activity of α_v integrins. Preferably, the integrin-inhibiting moiety inhibits the biological activity of the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; more preferably of the integrin $\alpha_v\beta_3$.

[0064] Integrin-inhibiting moieties may act as integrin-antagonists through any of a wide variety of mechanisms, and inhibition of the biological activity of integrins may therefore result from a large range of different processes affecting the integrins in a direct or indirect manner. For example, an integrin-inhibiting moiety may alter cell-matrix and cell-cell adhesion by inducing alterations in the integrin conformation and/or in the integrin cytoskeleton, or by causing disassociation of the α and β subunits (or any of the parts thereof) or disassociation of integrin clusters and/or clusters formed between integrins and other proteins.

[0065] An integrin-inhibiting moiety may also act directly on the receptor by blocking the binding to the integrin extracellular region, for example, by binding to a portion of at least one subunit (α or β) of the integrin. Preferably, integrin-inhibiting moieties inhibit the biological activity of integrins by binding to integrins. In certain preferred embodiments, integrin-inhibiting moieties have a high binding affinity and specificity for integrins. Preferably, integrin-inhibiting moieties have a high binding affinity and specificity for α_v integrins; more preferably for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; most preferably for the integrin $\alpha_v\beta_3$. More specifically, these integrin-inhibiting moieties bind α_v integrins with a submicromolar dissociation constant (K_d).

[0066] The interaction between an integrin-inhibiting moiety and integrins may be covalent or non-covalent. Most often, the interaction between an integrin-inhibiting moiety and integrins is non-covalent. Examples of non-covalent interactions include, but are not limited to, hydrophobic interactions, electrostatic interactions, dipole interactions, van der Waals interactions, and hydrogen bonding. Irrespective of the nature of the interaction, the binding between integrins and an integrin-inhibiting moiety in a bifunctional molecule should be selective, specific, and strong enough to allow the metal-chelating moiety to play its role (*i.e.*, to preclude, inhibit, reverse or otherwise interfere with the interactions between copper II and the metal-requiring angiogenic stimulators).

[0067] Suitable integrin-inhibiting moieties for use in the present invention include any entity capable of inhibiting the biological activity of integrins and that fulfill the requirements listed above. Actually, since most of the efforts in the development of anti-angiogenic agents have centered on integrin-antagonists, a large number of such compounds are now available.

[0068] For example, antibodies of a wide variety of integrins have been developed and shown to inhibit Matrigel™ invasion of glioma cell lines and primary cultures (W. Paulus and J.C. Tonn, J. Neurosurg. 1994, 80: 515-519). Vitaxin™ is an anti-$\alpha_v\beta_3$ monoclonal antibody that is currently in Phase II clinical trials. In phase I trials, Vitaxin™ produced stabilization of the disease or tumor shrinkage in 8 of 14 patients with no evidence of toxicity, even when given over 22 months (B.P. Eliceiri and D.A. Cheresh, J. Clin. Invest. 1999, 103: 1227-1230).

[0069] In certain preferred embodiments, integrin-inhibiting moieties are antibodies of integrins that have been reported to inhibit the biological activity of integrins.

[0070] Some integrins recognize certain matrix extracellular protein ligands that contain the RGD tripeptide sequence. For example, the $\alpha_v\beta_3$ receptor recognizes fibronectin, fibrinogen, vitronectin, laminin, von Willebrand factor, and osteopontin (R.I. Clyman *et al.*, Exp. Cell. Res. 1992, 200: 272-284; S.J. Shattil, Tromb. Haemostasis, 1995, 74: 149-155). More precisely, integrins are capable of distinguishing the different RGD-containing proteins thus showing different specificity for various extracellular ligands. It was found that the specificity of the RGD-integrin interaction results from variations in the RGD-conformation in the different proteins (involving not only the side chains but also the peptide backbone) as well as from contributions from sequences and residues adjacent to the RGD motif (see, for example, E. Ruoslahti and M.D. Pierschbacher, Science, 1987, 238: 491-497; M. Pfaff *et al.*, J. Biol. Chem. 1994, 269: 20233-20238).

[0071] A number of soluble cyclic or non-cyclic RGD-containing peptides have been designed, synthesized and tested for their ability to act as integrin-antagonists. For example, incorporation of this sequence in cyclic penta- and hexa-peptides containing D-amino acids, in a systematic manner, resulted in the development of highly potent and selective inhibitors of integrins. N-methylation or glycosylation of

some of these RGD-peptide derivatives has led to even more selective and active $\alpha_v\beta_3$ integrin antagonists. A discussion of the design of small molecule peptide-type agents that are $\alpha_v\beta_3$-selective inhibitors can be found in recent reviews (see, for example, A. Giannis and F. Ruebsam, Angew. Chem. Int. Ed. Eng. 1997, 36: 588-590).

[0072] RGD-containing peptides and RGD-based synthetic peptidomimetics that can be used in the present invention include, for example, those described in: M. Aumailly *et al.*, FEBS Lett. 1991, 291: 50-54; M. Gurrath *et al.*, Eur. J. Biochem. 1992, 210: 911-921; C.E. Peishoff *et al.*, J. Med. Chem. 1992, 35: 3962-3969; M. Pfaff *et al.*, J. Biol. Chem. 1994, 269: 20233-20238; R. Haubner *et al.*, J. Am. Chem. Soc. 1996, 118: 7461-7472; A.C. Bach *et al.*, J. Am. Chem. Soc. 1996, 118: 293-294; K. Burgess and D. Lim, J. Med. Chem. 1996, 39: 4520-4526; J. Wermuth *et al.*, J. Am. Chem. Soc. 1997, 119: 1328-1335; T.-A. Tran *et al.*, Bioorg. Med. Chem. Lett. 1997, 7: 997-1002; W.J. Hoekstra and B.L. Poulter, Curr. Med. Chem. 1998, 5: 195-204; C. P. Carron *et al.*, Cancer Res. 1998, 58: 1930-1935; J.S. Kerr *et al.*, Anticancer Res. 1999, 19: 959-968; M.A. Dechantsreiter *et al.*, J. Med. Chem. 1999, 42: 3033-3040; X. Dai *et al.*, Tetrahedron Lett. 2000, 41: 6295-6298; U.S. Pat. No. 5,780,426; D.L. Boger *et al.*, J. Am. Chem. Soc. 2001, 123: 1280-1288.

[0073] Numerous non-peptide mimetics containing different central scaffolds and exhibiting high affinity for the $\alpha_v\beta_3$ integrin and/or the $\alpha_v\beta_5$ integrin have been reported (as reviewed in: R. Mazitschek *et al.*, Mini-Rev. Med. Chem. 2002, 2: 491-506). These anti-angiogenic agents include those with indazole (D.G. Batt *et al.*, J. Med. Chem. 2000, 43: 41-58); benzene (M.E. Duggan *et al.*, J. Med. Chem. 2000, 43: 3736-3745); isoxazoline (W.J. Pitts *et al.*, J. Med. Chem. 2000, 43: 27-40; A.L. Rockwell *et al.*, Bioorg. Med. Chem. Lett. 1999, 9: 937-942); benzodiazepine (R.M. Keenan *et al.*, J. Med. Chem. 1997, 40: 2289; R.M. Keenan *et al.*, Bioorg. Med. Chem. Lett. 1999, 9: 1801-1806; R.M. Keenan *et al.*, Bioorg. Med. Chem. Lett. 1998, 8: 3171-3176; W.H. Miller *et al.*, J. Med. Chem. 2000, 43: 22-26); hydantoin (A. Peyman *et al.*, Bioorg. Med. Chem. Lett. 2000, 10: 179-182); benzazepine, benzodiazepine or benzocycloheptene (WO 96/00574, WO 96/00730, WO 96/06087, WO 96/26190, WO 97/24119, WO 97/24122, WO 97/24124, WO 99/05107, WO 99/06049, WO 99/15170, and WO 99/15178 assigned to SmithKline Beecham, and WO 97/34865 to Genentech, Inc.); and dibenzocycloheptene or dibenzoxazepine (WO

97/01540, WO 98/30542, WO 99/11626, and WO 99/15508 all assigned to SmithKline Beecham) scaffolds.

[0074] Other $\alpha_v\beta_3$ integrin antagonists have been described: for example, tyrosine derivatives (WO 97/23451 to Merck & Co., Inc.); sulfotyrosine derivatives (WO 97/45447); meta-guanidine, urea, thiourea or azacyclic amino benzoic acid derivatives (WO 97/08145 to Searle & Co.).

[0075] Other small molecule integrin-inhibitors include those described in: WO 97/37655; WO 98/08840; WO 98/18460; WO 98/18461; WO 98/31359; WO 99/30709; WO 99/30713; WO 99/31099; U.S. Pat. Nos. 5,919,792; 5,925,655 and 5,981,546 (all assigned to Merck & Co., Inc.); WO 98/25892 (to Eli Lilly & Co.); EP 853084; U.S. Pat. No. 6,011,045 (to Genentech, Inc.); and WO 97/36858 (to Searle & Co.).

[0076] In certain preferred embodiments, integrin-inhibiting moieties are RGD-containing peptides, RGD-based synthetic peptidomimetics, or small molecules that have been reported to exhibit a high affinity and specificity for α_v integrins, preferably for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins, most preferably for the integrin $\alpha_v\beta_3$.

[0077] Preferably, the integrin-inhibiting moieties contain at least one functional group that can be used (or can be easily chemically converted to a different functional group that can be used) to covalently attach the integrin-inhibiting moieties to other moieties (*e.g.*, metal-chelating moieties, imaging moieties, or boron-containing moieties). Suitable functional groups include, but are not limited to, amines (preferably primary amines), thiols, carboxy groups, and the like.

Metal-chelating Moieties

[0078] It has been known for some time that copper, which is the third most abundant trace metal in the human body, following iron and zinc, is essential to the strength and flexibility of skin, vasculature, and connective tissue. However, in the past three decades, *in vitro* and *in vivo* studies have also demonstrated the importance of copper in angiogenesis. Thus, the availability of copper *in vivo* has been shown to be critical to the initiation and development of some angiogenic processes (P. Gullino, Anticancer Res. 1986, 6: 326-335). Copper was also found to stimulate the proliferation and migration of human endothelial cells (G.F. Hu, J. Cell Biochem.

1998, 69: 326-335: B.R. McAuslan and W. Reilly, Exp. Cell Res. 1980, 130: 147-157). For numerous human cancers such as Hodgkin's lymphoma, sarcoma, leukemia, and cancer of the cervix, breast, liver, and lung (M. Diez *et al.*, Cancer, 1989, 63: 726-730; R.J. Coates *et al.*, Cancer Res. 1989, 49: 4353-4356; S.K. Gupta *et al.*, J. Surg. Oncol. 1991, 46: 178-181; S.K. Gupta *et al.*, J. Surg. Oncol. 1993, 52: 172-175; V.A. Senra-Varela *et al.*, Cancer Lett. 1997, 121: 139-145) as well as brain tumors (D. Yoshida *et al.*, J. Neuro-Oncol. 1993, 16: 109-115), tumor incidence, progression, severity and relapse are all associated with high levels of copper serum.

[0079] The role of copper in cancer promotion through inflammation and angiogenesis is now well understood. Copper was demonstrated to mediate the "switch" of the normally quiescent endothelium into a proliferative state by activation of angiogenic promoters (S. Brem, AACR Special Conference: Angiogenesis and Cancer Research, Jan. 24-28, 1998, Abstract A-16). More specifically, copper has been found to act as co-factor to key angiogenic stimulators such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and angiogenin. Without copper, these proteins do not function properly and the growth of new blood vessels stops. Similarly, endogenous copper-binding molecules (such as ceruloplasmin, which is the main copper carrier protein; heparin; and the tripeptide glycyl-hystidyl-lysine) are non-angiogenic when free of copper, but were found to become angiogenic when bound to copper (M. Ziche *et al.*, J. Natl. Cancer Inst. 1982, 69: 475-482).

[0080] Several studies showed a decrease in microvessel density and tumor size in penicillamine-treated, copper-deficient rabbits and rats xenografted with 9L gliosarcoma cells (S.S. Brem *et al.*, Am. J. Pathol. 1990, 137: 1121-1142; D. Yoshida *et al.*, J. Neuro-Oncol. 1993, 16: 109-115). Copper deficiency induced by tetrathiomolybdate significantly inhibited tumor growth of head and neck squamous cell carcinoma in severe combined immunodeficient mice (C. Cox *et al.*, Laryngoscope, 2001, 111: 696-701). Captopril, another copper-chelator (S.A. Müller *et al.*, Pharmacol. 1999, 58: 270-280), which is already widely used to treat non-malignant disease, was demonstrated to antagonize several steps of the angiogenesis cascade, resulting in decreased tumor growth (O.V. Volpert *et al.*, J. Clin. Invest. 1996, 98: 671-679). These results as well as preliminary data from clinical trials using penicillamine, tetrathiomolybdate and captopril show that the use of copper-

chelating agents may allow to fight multiple types of cancer by targeting copper as a "common denominator" to several of the key factors that activate the angiogenic process.

[0081] Therefore, by interfering with the interaction(s) between copper and some metal-requiring angiogenic stimulators, these metal-chelating molecules have a direct inhibiting effect on the angiogenic process. According to this aspect of the invention, suitable metal-chelating moieties are entities that can prevent or inhibit angiogenesis, by inhibiting the biological activity of copper-requiring angiogenic stimulators by precluding, reversing or otherwise interfering with the binding of copper to these angiogenic stimulators. More specifically, a suitable metal-chelating moiety for use in the present invention is any entity that exhibits a high binding affinity and specificity for copper II.

[0082] In certain preferred embodiments, metal-chelating moieties are selected from the group consisting of penicillamine, tetrathiomolybdate, captopril, and combinations thereof. Metal-chelating moieties for use in the present invention may also be functional derivatives, analogues and homologues of penicillamine, tetrathiomolybdate, or captopril.

[0083] Iron is also known to be a nutrient to cancer, to promote inflammation, to increase cancer cell growth and to favor free-radical activity. Iron overload conditions are associated with increased incidence of cancer, heart disease and defective immune regulatory control (E.M. Walker, Annals of Clinical & Laboratory Science, 2000, 30: 354-365) and recently, it was also found that an excess of iron may actually stimulate angiogenesis (K. Norrby *et al.*, Int. J. Cancer, 2001, 91: 236-40).

[0084] Accordingly, suitable metal-chelating moieties for use in the present invention are entities that can prevent or inhibit angiogenesis, by binding with high affinity copper II, iron III, or both. Preferably, metal-chelating moieties are stable, non-toxic entities that retain their binding properties under *in vitro* and *in vivo* conditions.

[0085] Therefore, suitable metal-chelating moieties may be any metal chelator and metal complexing molecule that binds with high affinity copper II, and/or iron III. These include, but are not limited to, aromatic amines such as bathophenanthroline (4,7-diphenyl-1,10-phenanthroline); bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-

phenanthroline), and TPEN (tetrakis-(2-pyridylmethyl) ethylenediamine); and aliphatic amines such as deferrioxamine, EDTA (ethylenediamine-tetraacetic acid), EGTA (O,O'-bis(2-aminoethyl)-ethyleneglycol-N,N',N",N""-tetraacetic acid), DTPA (diethylene-triaminepentaacetic acid), and TETA (triethylene tetramine); and functional derivatives, homologues and analogues thereof.

[0086] Preferably, the metal-chelating moieties contain at least one functional group that can be used (or can be easily chemically converted to a different functional group that can be used) to covalently attach them to integrin-inhibiting moieties. Suitable functional groups include, but are not limited to, amines (preferably primary amines), thiols, carboxy groups, and the like.

Synthesis of Bifunctional Molecules

[0087] The inventive bifunctional molecules may be prepared by any available synthetic method, the only requirement being that, after reaction, the integrin-inhibiting and metal-chelating moieties retain their respective properties. The integrin-inhibiting moieties may be associated with the metal-chelating moieties in a variety of ways. Preferably, the integrin-inhibiting moieties are covalently attached to the metal-chelating moieties. As can be appreciated by those skilled in the art, the integrin-inhibiting and metal-chelating moieties may be attached to each other either directly or through a linker.

[0088] In certain preferred embodiments, the metal-chelating and integrin-inhibiting moieties are directly covalently linked to each other. The direct covalent binding can be through an amide, ester, carbon-carbon, disulfide, carbamate, ether, thioether, urea, amine, or carbonate linkage. The covalent binding can be achieved by taking advantage of functional groups present on the integrin-inhibiting and metal-chelating moieties. Suitable functional groups that can be used to attach the two moieties together include, but are not limited to, amines (preferably primary amines), anhydrides, hydroxy groups, carboxy groups, and thiols. For example, as described in Example 1, an amide bond may be formed by reaction between the primary amino group present on the integrin-inhibiting moiety and the anhydride function on the metal-chelating moiety. A direct linkage may also be formed by using an activating agent, such as a carbodiimide, to bind, for example, the primary amino group present

on one moiety to the carboxy group present on the other moiety. A wide range of activating agents are known in the art and are suitable for use in the present invention.

[0089] In other preferred embodiments, the metal-chelating and integrin-inhibiting moieties are indirectly covalently linked to each other *via* a linker group. This can be accomplished by using any number of stable bifunctional agents well known in the art, including homofunctional and heterofunctional linkers (see, for example, Pierce Catalog and Handbook, 1994). The use of a bifunctional linker differs from the use of an activating agent in that the former results in a linking moiety being present in the inventive bifunctional molecule after reaction, whereas the latter results in a direct coupling between the two moieties involved in the reaction. The main role of the bifunctional linker is to allow the reaction between two otherwise inert moieties. However, the bifunctional linker, which becomes part of the reaction product, can also be selected such that it confers some degree of conformational flexibility to the bifunctional molecule (*e.g.*, the bifunctional linker comprises a straight alkyl chain containing several atoms, for example the straight alkyl chain contains between 2 and 10 carbon atoms).

[0090] A wide range of suitable homofunctional and heterofunctional linkers known in the art can be used in the context of the present invention. Preferred linkers include, but are not limited to, alkyl and aryl groups, including straight chain and branched alkyl groups, substituted alkyl and aryl groups, heteroalkyl and heteroaryl groups, that have reactive chemical functionalities such as amino, anhydride, hydroxyl, carboxyl, carbonyl groups, and the like.

[0091] As can readily be appreciated by those skilled in the art, a bifunctional molecule of the invention can comprise any number of integrin-inhibiting moieties and any number of metal-chelating moieties, linked to one another by any number of different ways. The integrin-inhibiting moieties within an inventive bifunctional molecule may be all identical or different. Similarly, the metal-chelating moieties within an inventive bifunctional molecule may be all identical or different. The design of a bifunctional therapeutic molecule will be influenced by its intended purpose(s) and the properties that are desirable in the particular context of its use.

II. Targeted Contrast Imaging Agents

[0092] Another aspect of the invention relates to a new class of targeted contrast imaging agents for the imaging of angiogenesis in *in vitro*, *in vivo*, and *ex vivo* systems as well as in patients.

[0093] As already mentioned above, the lack of adequate methods for monitoring treatment and drug effects in patients with pathophysiological conditions associated with angiogenesis constitutes a major impediment to the development of new therapies and therapeutic agents. Therefore new or improved procedures for the imaging of angiogenesis are highly desirable. An attractive approach is to target specific markers of the angiogenic disease, such as those that are highly expressed on proliferating endothelial cells. Thus, an ideal probe for the imaging of angiogenesis would be one that exhibits a high affinity for a specific marker of the disease; has a low toxicity and allows for the non-invasive imaging, detection, localization and quantification of angiogenesis in live patients.

[0094] The present invention is directed to targeted, detectable reagents that meet at least some of the criteria listed above. Accordingly, the present invention provides targeted contrast imaging agents that are designed to (1) have some degree of attraction for integrins, and (2) be detectable by imaging techniques. More specifically, the invention provides contrast imaging agents comprising at least one imaging moiety associated with at least one integrin-binding moiety.

Integrin-Binding Moieties

[0095] Integrin-binding moieties in the contrast imaging agents of the invention are targeting entities that display some degree of attraction for integrins, *i.e.*, they specifically and efficiently interact with, and bind to integrins. Suitable integrin-binding moieties for use in the design and development of contrast imaging agents are therefore the integrin-inhibiting moieties described and listed above that act as integrin-antagonists by precluding, reversing, inhibiting or otherwise interfering with the binding of integrins with their endogenous ligands.

[0096] In certain preferred embodiments, the integrin-binding moieties exhibit a high binding affinity and specificity for integrins. In other preferred embodiments, the integrin-binding moieties have a high binding affinity and specificity for α_v integrins; preferably for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; more preferably for the $\alpha_v\beta_3$ integrin.

Imaging Moieties

[0097] In the context of the present invention, imaging moieties are entities that are detectable by imaging techniques such as Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS), Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET). Preferably, imaging moieties are stable, non-toxic entities that retain their properties under *in vitro* and *in vivo* conditions.

[0098] *MRI Imaging Moieties.* In certain preferred embodiments, the contrast imaging agents of the invention are designed to be detectable by Magnetic Resonance Imaging.

[0099] MRI has evolved into one of the most powerful non-invasive techniques in diagnostic clinical medicine and biomedical research (P. Caravan *et al.*, Chem. Rev. 1999, 99: 2293-2352; M.M. Huber *et al.*, Bioconjug. Chem. 1998, 9: 242-249; W.H. Li *et al.*, J. Am. Chem. Soc. 1999, 121: 1413-1414; X. Yu *et al.*, Mag. Res. Med. 2000, 44: 867-872). MRI is an application of Nuclear Magnetic Resonance (NMR), a well known analytical method used in chemistry, physics and molecular structural biology. MRI can generate three dimensional structural information in relatively short time spans and is widely used as a non-invasive diagnostic tool to identify potentially maleficent physiological anomalies, to observe blood flow or to determine the general status of a cardiovascular system.

[0100] MRI has the advantage (over other high-quality imaging methods) of not relying on potentially harmful ionizing radiation (A.R. Johnson *et al.*, Inorg. Chem. 2000, 39: 2652-2660). In MRI, a contrast image of a biological sample or patient's body is provided by monitoring local variations in water concentrations and T_1 (spin-lattice) and T_2 (spin-spin) relaxation times of NMR signals from water protons (^1H). Often the clarity of MRI can be improved through the use of contrast imaging agents. Due to their paramagnetic properties, these agents decrease the T_1 and T_2 relaxation times by using their unpaired electrons to facilitate spin transfer. This results in an increase of concentration-dependent contrast and consequently an enhanced differentiation between anatomical structures.

[0101] Since the paramagnetic susceptibility of an entity (and hence its ability to shorten the T_1 and T_2 relaxation times of the proton nuclei of nearby water molecules)

increases with the number of unpaired electrons (F.A. Cotton *et al.*, "Basic Inorganic Chemistry", John Wiley & Sons, New York, 1995, p. 68), ideal paramagnetic metal ions for use in MRI should, in principle, have as many unpaired electrons as possible. However, the complex of such paramagnetic metal ions with water molecules are highly toxic and therefore useless for *in vivo* imaging. By complexing a paramagnetic metal ion to a ligand or metal-chelating moiety and leaving only one coordination site open for a water molecule, the toxicity has been found to be strongly reduced. Therefore, most MRI contrast agents typically consist of chelated paramagnetic metal ions.

[0102] Accordingly, in certain preferred embodiments of the invention, the MRI contrast imaging agents are designed such that the imaging moiety comprises at least one metal-chelating moiety complexed to a paramagnetic metal ion.

[0103] Suitable paramagnetic metal ions for use in the present invention include any of the paramagnetic metal ions known in the art to be physiologically acceptable, good contrast enhancers in MRI, and easily incorporated into metal-chelating moieties. Preferably, the paramagnetic metal ion is selected from the group consisting of gadolinium III (Gd^{3+}), chromium III (Cr^{3+}), dysprosium III (Dy^{3+}), iron III (Fe^{3+}), manganese II (Mn^{2+}), and ytterbium III (Yb^{3+}). More preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}). Gadolinium is an FDA-approved contrast agent for MRI, which accumulates in abnormal tissues causing these abnormal areas to become very bright (enhanced) on the magnetic resonance image. Gadolinium is known to provide great contrast between normal and abnormal tissues in different areas of the body, in particular in the brain.

[0104] Suitable metal-chelating moieties for use in the present invention include any entity capable of complexing paramagnetic metal ions detectable by MRI. Preferably, a metal-chelating moiety is a stable, non-toxic entity that binds a paramagnetic metal ion in such a way that it leaves one coordination site open for a water molecule and with such high affinity that, once complexed, the paramagnetic metal ion cannot be displaced by water.

[0105] A number of such metal-chelating moieties have been used for the complexation of Gd^{3+}. Those include DTPA; 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA); and derivatives thereof (see, for example, U.S.

Pat. Nos. 4,885,363; 5,087,440; 5,155,215; 5,188,816; 5,219,553; 5,262,532; 5,358,704; and D. Meyer *et al.*, Invest. Radiol. 1990, 25: S53-55). However, gadolinium complexes of these ligands are salts under physiological conditions, and the requirement of non-paramagnetic cationic counterions increases the osmolality of the solution. A neutral gadolinium complex that retains high water solubility and relaxativity has been prepared using DTPA-bis(amide) derivatives (U.S. Pat. No. 4,687,659).

[0106] Other metal-chelating moieties that complex paramagnetic metal ions include acyclic entities such as aminopolycarboxylic acids and phosphorus oxyacid analogues thereof (*e.g.*, triethylenetetramine-hexaacetic acid or TTHA, and dipyridoxal diphosphate, or DPDP) and macrocyclic entities (*e.g.*, 1,4,7,10-tetraazacyclododecane-N,N',N''-triacetic acid or DO3A). Metal-chelating moieties may also be any of the entities described in U.S. Pat. Nos. 5,410,043; 5,277,895 and 6,150,376; and F.H. Arnold, Biotechnol. 1991, 9: 151-156.

[0107] *MRS Imaging Moieties.* In certain preferred embodiments, the contrast imaging agents of the invention are designed to be useful in Magnetic Resonance Spectroscopy (MRS). More specifically, the present invention provides contrast imaging agents comprising at least one metal-chelating moiety associated with at least one integrin-inhibiting moiety labeled with a magnetically active nuclei. Preferred magnetically active nuclei are the natural isotopes carbon-13 (^{13}C) and fluorine-19 (^{19}F).

[0108] *Radioactive Imaging Moieties.* In other preferred embodiments, the contrast imaging agents of the invention are designed to be detectable by Single Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET).

[0109] SPECT and PET are nuclear medicine imaging techniques which have been used to detect tumors, aneurysms (weak spots in blood vessel walls), irregular or inadequate blood flow to various tissues, blood cell disorders, and inadequate functioning of organs, such as thyroid and pulmonary function deficiencies. Both techniques acquire information on the concentration of radionuclides introduced into a biological sample or to a patient's body. PET generates images by detecting the radiation emitted from short-lived radioactive substances, which are formed by

bombarding non-radioactive chemicals with neutrons to create radioactive isotopes. PET detects the gamma rays given off at the site where a positron emitted from the radioactive substance collides with an electron in the tissue. A PET analysis results in a series of thin slice images of the body over the region of interest (*e.g.*, brain, breast, liver). These thin slice images can be assembled into a three dimensional representation of the examined area. However, there are only few PET centers because they must be located near a particle accelerator device that is required to produce the short-lived radioisotopes used in the technique. SPECT is similar to PET, but the radioactive substances used in SPECT (*e.g.*, ^{99m}TC, ^{123}I, ^{133}Xe) have longer decay times than those used in PET and emit single instead of double gamma rays. Although SPECT images exhibit less sensitivity and are less detailed than PET images, the SPECT technique exhibits several advantages over PET in that it does not require the proximity of a particle accelerator and is much less expensive than PET.

[0110] Accordingly, in certain preferred embodiments, the contrast imaging agents of the invention are designed to be detectable by Single Photon Emission Computed Tomography (SPECT). Preferably, the imaging moiety in the contrast imaging agent comprises at least one metal-chelating moiety complexed to a metal entity that is detectable by SPECT.

[0111] Suitable metal entities for use in the present invention are radionuclides known in the art to be physiologically acceptable, detectable by SPECT, and easily incorporated into metal-chelating moieties. Preferably, the radionuclide is selected from the group consisting of technetium-99m (^{99m}Tc), gallium-67 (^{67}Ga), yttrium-91 (^{91}Y), indium-111 (^{111}In), rhenium-186 (^{186}Re), and thallium-201 (^{201}Tl). Most preferably, the radionuclide is technetium-99m (^{99m}Tc). Over 85% of the routine nuclear medicine procedures currently performed use radiopharmaceutical methodologies based on ^{99m}Tc.

[0112] Suitable metal-chelating moieties for use in the present invention include any stable and non-toxic entity that complexes short-lived radionuclides detectable by SPECT.

[0113] Metal-chelating moieties that complex radionuclides such as ^{99m}Tc are well known in the art (see, for example, "*Technetium and Rhenium in Chemistry and Nuclear Medicine*" M. Nicolini *et al.*, Eds., 1995, SGEditoriali: Padova, Italia).

Suitable metal-chelating moieties include, for example, N_2S_2 and N_3S chelators (A.R. Fritzberg *et al.*, J. Nucl. Med. 1982, 23: 592-598) which can complex a radionuclide through two nitrogen atoms and two sulfur atoms, or through three nitrogen atoms and one sulfur atom, respectively. Ethyl cysteine dimer (ECD) is an N_2S_2 chelator well known in the art. N_2S_2 and N_3S chelators are, for example, described in U.S. Pat. Nos. 4,444,690; 4,670,545; 4,673,562; 4,897,255; 4,965,392; 4,980,147; 4,988,496; 5,021,556 and 5,075,099.

[0114] Other suitable metal-chelating moieties can be selected from polyphosphates (*e.g.*, ethylenediaminetetramethylenetetraphosphonate, EDTMP); aminocarboxylic acids (*e.g.*, EDTA, N-(2-hydroxy)ethylene-diaminetriacetic acid, nitrilotriacetic acid, N,N-di(2-hydroxy-ethyl)-glycine, ethylenebis(hydroxyphenylglycine) and diethylenetriamine pentacetic acid); 1,3-diketones (*e.g.*, acetylacetone, trifluoroacetylacetone, and thenoyltrifluoroacetone); hydroxy-carboxylic acids (*e.g.*, tartaric acid, citric acid, gluconic acid, and 5-sulfosalicyclic acid); polyamines (*e.g.*, ethylenediamine, diethylenetriamine, triethylenetetraamine, and triamino-triethylamine); aminoalcohols (*e.g.*, triethanolamine and N-(2-hydroxyethyl)ethylenediamine); aromatic heterocyclic bases (*e.g.*, 2,2'-diimidazole, picoline amine, dipicoline amine and 1,10-phenanthroline); phenols (*e.g.*, salicylaldehyde, disulfopyrocatechol, and chromotropic acid); aminophenols (*e.g.*, 8-hydroxyquinoline and oximesulfonic acid); oximes (*e.g.*, hexamethyl-propyleneamine oxime, HMPAO); Schiff bases (*e.g.*, disalicylaldehyde 1,2-propylenediimine); tetrapyrroles (*e.g.*, tetraphenylporphin and phthalocyanine); sulfur compounds (*e.g.*, toluenedithiol, meso-2,3-dimercaptosuccinic acid, dimercaptopropanol, thioglycolic acid, potassium ethyl xanthate, sodium diethyldithiocarbamate, dithizone, diethyl dithiophosphoric acid, and thiourea); synthetic macrocyclic compounds (*e.g.*, dibenzo[18]crown-6), or combinations of two or more of the above agents.

[0115] Preferred metal-chelating moieties are selected from the group consisting of polycarboxylic acids such as EDTA, DTPA, DOTA, DO3A; and derivatives, homologues and analogues thereof, or combinations thereof.

[0116] Other suitable metal-chelating moieties are described in U.S. Pat. No. 5,559,214, WO 95/26754, WO 94/08624, WO 94/09056, WO 94/29333, WO 94/08624, WO 94/08629, WO 94/13327 and WO 94/12216.

[0117] Preferably, the metal-chelating moieties contain at least one functional group that can be used (or can be easily chemically converted to a different functional group that can be used) to covalently attach the metal-chelating moieties to integrin-inhibiting moieties. Suitable functional groups include, but are not limited to, amines (preferably primary amines), thiols, carboxy groups, and the like.

Synthesis of Contrast Imaging Agents

[0118] The inventive contrast imaging agents may be prepared by any available synthetic method, the only requirement being that, after reaction, the integrin-binding and imaging moieties retain their respective properties. The integrin-binding moieties may be associated with the imaging moieties in a variety of ways. Preferably, the integrin-binding moieties are covalently attached to the imaging moieties. As can be appreciated by those skilled in the art, the integrin-binding and imaging moieties may be attached to each other either directly or through a linker.

[0119] The methods described above to prepare the inventive bifunctional molecules can be used to synthesize the contrast imaging agents.

[0120] Imaging moieties that comprise at least one metal-chelating moiety complexed to a metal entity can be prepared by any method known in the art. Complexation may be carried out before, during or after formation of direct or indirect covalent bonds between the metal-chelating and integrin-inhibiting moieties. Preferably, the complexation is carried out using an inventive bifunctional molecule as starting material (see Examples 2 and 3). When the metal entity is a short-lived radionuclide, the complexation is carried out shortly before the contrast imaging agent is used.

[0121] Suitable complexation methods include, for example, direct incorporation of the metal entity into the metal-chelating moiety and transmetallation. When possible, direct incorporation is preferred. In this method, an aqueous solution of a metal-chelating moiety is generally exposed or mixed with a metal salt. The pH of the reaction mixture may be between about 4 and about 11. Preferably, the pH is between 5 and 9. More preferably, the reaction is carried out at a pH between 6 and 8. Direct incorporation methods are well known in the art and several procedures have been described (W. D'Olieslager and G.R. Choppin *et al.*, J. Inorg. Nucl. Chem. 1971, 33: 127-135; G.R. Choppin and K.R. Williams, J. Inorg. Nucl. Chem. 1973, 35:

4255-4269; WO 87/06229). Transmetallation is used when the metal entity needs to be reduced to a different oxidative state before incorporation. Transmetallation methods are well known in the art. Example 2 illustrates such a reaction, where the incorporation of ^{99m}Tc into a bifunctional molecule is carried out by reducing the metal ion to Tc(V) using $SnCl_2$.

[0122] As can be readily appreciated by those skilled in the art, a contrast imaging agent may comprise any number of integrin-binding moieties and any number of imaging moieties, linked to one another by any number of different ways. The integrin-binding moieties within a contrast imaging agent may be all identical or different. Similarly, the imaging moieties within a contrast imaging agent may be all identical or different. The precise design of a contrast imaging agent will be influenced by its intended purpose(s) and the properties that are desirable in the particular context of its use.

III. Targeted Boron-containing Compounds

[0123] One aspect of the present invention relates to a new class of targeted reagents that can be used as therapeutics in Boron Neutron Capture Therapy (or BNCT).

Boron Neutron Capture Therapy

[0124] Boron Neutron Capture Therapy is an experimental binary radiotherapy modality that is being tested for the treatment of cancers such as brain tumors (glioma and glioblastoma) and metastatic malignant melanoma. BNCT, which has the potential ability to selectively kill tumor cells while sparing normal healthy tissue, involves the preferential accumulation of a boron-10 (^{10}B)-containing chemical agent in a tumor and the subsequent irradiation of the boron-loaded tumor with a neutron radiation beam of adequate energy (*i.e.*, thermal or epithermal neutrons of less than 0.5 eV to about 30-100 keV). When used separately, the boron agent and the flux of neutrons fail to produce significant damage to tissues. However, the capture reaction between the stable isotope ^{10}B and neutrons leads to the formation of heavy particles that damage tumor cells and induce tumor shrinkage. The short range (*i.e.*, pathlength) in tissue of the emitted particles, α and ^{7}Li, (< 10 μm, which is

comparable to or less than the diameter of a cell) allows localized energy release and in principle, only the cells in which the fission reaction takes place are destroyed.

[0125] BNCT takes advantage of the preferential localization of the boron agent in tumors compared with healthy tissues and of the high cross section (localized range of action) of the nuclear reaction of neutrons with ^{10}B. Ideal boron agents for use in BNCT are therefore compounds that (1) are able to deliver a large amount of Boron atoms to tumor sites, while leaving non-cancerous sites relatively boron-free, (2) exhibit a sustained retention by tumor tissues, and (3) are capable of crossing the blood-brain barrier to reach even microscopic extensions of tumors. The boron agents currently in use (*e.g.*, boronphenylalanine-fructose (BPA) and sodium mercaptoborate (BSH, $Na_2{}^{10}B_{12}H_{11}SH$)) are not ideal compounds for BNCT.

[0126] The present invention provides reagents that are useful in BNCT and exhibit at least some of the ideal properties listed above. In particular, the present invention encompasses reagents that can be used as therapeutics in BNCT and exhibit a high selectivity for tumor tissues by targeting endothelial cells, more specifically by binding to integrins. By targeting endothelial cells that lie in direct contact with blood (instead of malignant cells that are often sequestered in body compartment), these reagents can directly reach their targets without being affected by delivery issues. Therefore, the blood-brain barrier, which is a formidable obstacle to many boron agents targeted to malignant cells, is irrelevant in the case of the inventive reagents.

[0127] More specifically, the invention provides boron-containing compounds comprising at least one integrin-binding moiety associated with at least one boron-containing moiety. The invention also provides boron-containing compounds containing at least one metal-chelating moiety associated with at least one integrin-binding moiety labeled with Boron-10. Other boron-containing compounds of the invention comprise at least one imaging moiety associated with at least one integrin-binding moiety labeled with Boron-10.

Integrin-binding Moieties

[0128] Integrin-binding moieties in the boron-containing compounds of the invention play the same role than in the contrast imaging agents described above: they are targeting entities that display dome degree of attraction for integrins, *i.e.*, they specifically and efficiently interact with and bind to integrins. Suitable integrin-

binding moieties for use in the design and development of boron-containing compounds are therefore the integrin-inhibiting moieties listed above that act as integrin-antagonists by precluding, reversing, inhibiting or otherwise interfering with the binding of integrins with their endogenous ligands.

[0129] In certain preferred embodiments, the integrin-binding moieties exhibit a high binding affinity and specificity for integrins. In other preferred embodiments, the integrin-binding moieties have a high binding affinity and specificity for α_v integrins; preferably for the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; more preferably for the $\alpha_v\beta_3$ integrin.

Boron-containing Moieties

[0130] A boron-containing moiety is any entity that can be used in BNCT, *i.e.*, that produces α particles inducing biological damage and tumor shrinkage when irradiated with a neutron radiation beam of adequate energy. Preferably, boron-containing moieties are stable, non-toxic entities that retain their properties under *in vitro* and *in vivo* conditions. Preferably, boron-containing moieties are entities that, when associated with integrin-binding moieties, become selectively targeted to integrins (in other words, when not associated with integrin-binding moieties, boron-containing moieties are not, themselves, targeted entities). Preferred boron-containing moiety contains at least a natural abundance (*i.e.*, ~ 19%), preferably at least about 99% of Boron-10.

[0131] Suitable boron-containing moieties for use in the present invention include compounds comprising a single boron atom per molecule, such as boric acid, as well as compounds containing more than one boron atom per molecule. For example, boron-containing moieties may be polyhedral boron anions such as closo-$B_{12}H_{12}^{2-}$ and closo-$B_{10}H_{10}^{2-}$ (M.F. Hawthorne *et al.*, J. Am. Chem. Soc. 1959, 81: 5519; A. Lipscomb, J. Am. Chem. Soc. 1959, 81: 5833-5834) and their derivatives such as BSH ($B_{12}H_{12}SH^{2-}$ described by W. Knoth, J. Am. Chem. Soc. 1964, 86: 3973-3983).

[0132] Suitable boron-containing moieties can also be compounds that include two polyhedral borane anion cages linked together to form a linked cage structure comprising, for example, 20 boron atoms, such as those described by M.F. Hawthorne, Angew. Chem. Int. Ed. Engl. 1993, 32: 950-954.

[0133] Boron-containing moieties can also be based on polyhedral carboranes such as compounds of the general formula closo-$C_2B_{n-2}H_n$ (J. Grafstein, Inorg. Chem. 1963, 2: 1128-1133; J. Morris *et al.*, in "*Progress in Neutron Capture Therapy of Cancer*", B. Allen *et al.*, (Eds), Plenum Press, New York, 1992, p. 215), and their derivatives (U.S. Pat. Nos. 6,086,637 and 6,143,264).

[0134] Other suitable boron-containing moieties are boronated derivatives of porphyrins (S.B. Kahl and M.-S.Koo, in: "*Progress in Neutron Capture Therapy for Cancer*", B.J. Allen *et al.*, (Eds), Plenum Press, New York, 1992, pp. 223-226; U.S. Pat. Nos. 4,959,356; 5,149,801 and 5,877,165), metalloporphyrins (U.S. Pat. No. 5,654,423) and texaphyrins (U.S. Pat. No. 5,955,586); radiation sensitizers (U.S. Pat. No. 5,405,598); polyamines (J.R. Hariharan *et al.*, Polyhedron, 1995, 14: 823-825); purines and pyrimidines (U.S. Pat. No. 5,362,732).

[0135] Preferably, the boron-containing moieties contain at least one functional group that can be used (or can be easily chemically converted to a different functional group that can be used) to covalently attach the boron-containing moieties to integrin-binding moieties. Suitable functional groups include, but are not limited to, amines (preferably primary amines), thiols, carboxy groups, and the like.

Integrin-Binding Moieties Labeled With Boron-10

[0136] Integrin-binding moieties labeled with Boron-10 play two different roles: they (1) are targeting entities that display some degree of attraction for integrins (*i.e.*, they specifically and efficiently interact with and bind to integrins) and (2) have a therapeutic action, since upon irradiation with a beam of neutrons of adequate energy, the reaction involving ^{10}B atoms induces biological damage and tumor shrinkage.

Suitable integrin-binding moieties for use in the design and development of inventive boron-containing compounds are therefore the integrin-inhibiting moieties listed above that act as integrin-antagonists by precluding, reversing, inhibiting or otherwise interfering with the binding of integrins with their endogenous ligands and that can be labeled with Boron-10.

Metal-chelating Moieties

[0137] Metal-chelating moieties in the boron-containing compounds play the same role than in the bifunctional molecules of the invention described above: they

bind with high affinity transition metal ions that are involved in one or more steps of the angiogenic process. Preferably, the metal-chelating moieties bind with high affinity and specificity copper II. Suitable metal-chelating moieties for use in the design and development of boron-containing compounds are therefore those listed above.

Imaging Moieties

[0138] The presence of imaging moieties in the boron-containing compounds of the invention allows the determination of the location of the administered compound by imaging techniques. When a boron-containing compound comprises one or more imaging moieties, it can be detected before the boron is submitted to the neutron radiation beam to ensure that the compound has localized in the tumor and that non-localized boron-containing compound has cleared from the circulation. This can help minimize the risks of damaging healthy tissues when the boron is irradiated, as irradiation can be delayed until the boron compound has fully localized at tumor site(s). The detection of the boron-containing compound can also help in the planning of the BNCT treatment (such as predicting normal tissue tolerances, predicting or modeling possible tumor response), and especially in the microdosimetric measurements and calculations that are necessary for the determination of the irradiation dose to be administered.

[0139] Imaging moieties in the boron-containing compounds play the same role than in the contrast imaging agents of the invention, they render the molecule detectable by an imaging technique. In certain preferred embodiments, imaging moieties in boron-containing compounds comprise at least one metal-chelating moiety complexed to paramagnetic metal ion that is detectable by MRI. Preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}). In other preferred embodiments, imaging moieties in boron-containing compounds comprise at least one metal-chelating moiety complexed to a radionuclide that is detectable by SPECT. Preferably, the radionuclide is technetium-99m (^{99m}Tc). Suitable imaging moieties for use in the design and development of boron-containing compounds are therefore those listed above.

Synthesis of Targeted Boron-containing Compounds

[0140] The inventive boron-containing compounds may be prepared by any available synthetic method, the only requirement being that, after reaction, the different moieties comprised in the boron-containing compound retain their respective properties. The integrin-binding moieties may be associated with the boron-containing moieties in a variety of ways. Preferably, the integrin-binding moieties are covalently attached to the boron-containing moieties. As can be appreciated by those skilled in the art, the integrin-binding and boron-containing moieties may be attached to each other either directly or through a linker.

[0141] The methods described above to prepare the bifunctional therapeutic molecules and targeted contrast imaging agents can be used to synthesize the boron-containing compounds.

[0142] The preparation of boron-containing moieties may be carried out by any suitable method known in the art. Preparations of boron organic compounds are, for example, described in: Hagihara *et al.*, "*Handbook of Organometallic compounds*", Chapter 3, W.A. Benjamin, Inc., New York, 1968; and Wong *et al.*, J. Med. Chem. 1974, 17: 785-791.

[0143] As can be readily appreciated by those of ordinary skill in the art, a boron-containing compound of the invention may comprise any number of integrin-binding moieties and any number of boron-containing moieties, linked to one another by any number of different ways. The integrin-binding moieties within a boron-containing compound may be all identical or different. Similarly, the boron-containing moieties within a boron-containing compound may be all identical or different. The precise design of a boron-containing compound will be influenced by its intended purpose(s) and the properties that are desirable in the particular context of its use.

IV. Uses of the Bifunctional Therapeutic Molecules

[0144] Another aspect of the present invention relates to strategies for preventing or inhibiting angiogenesis. Accordingly, the present invention provides reagents and strategies that allow for the prevention or inhibition of the angiogenic process by inhibiting the biological activity of integrins. The present invention also provides reagents and strategies for preventing or inhibiting the angiogenic process by

inhibiting the biological action of angiogenic stimulators that require the presence of transition metal ions as co-factors.

[0145] More specifically, the present invention provides reagents that act both as integrin-antagonists and metal chelators, and methods of using them for preventing or inhibiting angiogenesis in *in vitro*, *in vivo* and *ex vivo* systems as well as in patients. The methods provided herein comprise using bifunctional molecules of the invention that have a dual mode of action.

[0146] In certain preferred embodiments, the invention allows the prevention or inhibition of angiogenesis by inhibiting the biological activity of integrins. Preferably, the inventive methods prevent or inhibit angiogenesis by inhibiting the biological activity of α_v integrins; more preferably of the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins; most preferably, of the $\alpha_v\beta_3$ integrins.

[0147] In other preferred embodiments, the invention allows the prevention or inhibition of angiogenesis by inhibiting the biological action of angiogenic stimulators that require the presence of transition metal ions as co-factors. Preferably, the inventive methods prevent or inhibit angiogenesis by precluding, reversing, disrupting or otherwise interfering with the interaction of these angiogenic stimulators with transition metal ions. More preferably, the inventive methods prevent or inhibit angiogenesis by precluding, reversing, disrupting or otherwise interfering with the interaction between the angiogenic stimulators and copper II. This can be achieved when the metal-chelating moiety in the bifunctional molecule binds transition metal ions, preferably when the metal-chelating moiety in the bifunctional molecule binds copper II with high affinity and specificity.

[0148] More specifically, the present invention provides methods for preventing or inhibiting angiogenesis in a system, comprising contacting the system with an effective amount of a bifunctional molecule of the invention, or a pharmaceutical composition thereof.

[0149] The contacting may be carried out *in vitro*, *in vivo*, or *ex vivo*. For example, the contacting may be carried out by incubation.

[0150] The system may be any biological entity known in the art to be able to undergo angiogenesis. For example, the system may be a cell, a biological fluid, a biological tissue, or an animal. When the system is a cell, a biological fluid or a

biological tissue, it may originate from a live patient (*e.g.*, it may be obtained by biopsy) or a deceased patient (*e.g.*, it may be obtained at autopsy). The patient may be a human or another mammal. In preferred embodiments, the cell, biological fluid, or biological tissue originates from a patient suspected to have a pathophysiological condition associated with angiogenesis. In other preferred embodiments, the cell, biological fluid, or biological tissue originates from a patient suspected to have a pathophysiological condition selected from the group consisting of cancer, psoriasis, atherosclerosis, restenosis, rheumatoid arthritis, and ocular neovascularization (leading to diabetic retinopathy, neovascular glaucoma, age-related macular degeneration, or retinal vein occlusion).

[0151] The present invention also provides methods for treating a patient with a pathophysiological condition associated with angiogenesis. The methods described herein may be carried out (1) to delay or prevent the onset of the disease; or (2) to slow down or stop the progression, aggravation, or deterioration of the disease; or (3) to reverse or bring about ameliorations of the symptoms and signs of the disease; or (4) to cure the disease. The treatment may be administered prior to the onset of the disease, for a prophylactic or preventive action, or after initiation of the disease, for a therapeutic action. In case of cancer, the treatment may, for example, be administered to prevent the formation of metastases.

[0152] More specifically, the present invention provides methods for treating a patient with a pathophysiological condition associated with angiogenesis, comprising administering to the patient an effective amount of a bifunctional molecule of the invention, or a pharmaceutical composition thereof.

[0153] Administration of the bifunctional molecule, or pharmaceutical composition thereof, may be performed by any suitable method known in the art, for example, by oral and parenteral administrations, (*i.e.*, intravenous, intramuscular, or subcutaneous injection), transdermal and topical administrations.

[0154] The pathophysiological condition associated with angiogenesis may affect any organ or tissue of the body, and may or may not be localized. The pathophysiological condition that can be treated by the inventive methods may be any of the angiogenic diseases and disorders known in the art to be associated with an up-regulation of the angiogenic process leading to excessive proliferation of blood

vessels. As angiogenic inhibitors, the bifunctional molecules of the invention may be useful to treat a wide variety of clinical conditions. These conditions include, for example, primary and metastatic solid tumors and carcinomas of the breast; lung; gastro-intestinal tract; urinary tract; female genital tract; male genital tract; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumors of the brain (*e.g.*, gliomas, gliobastomas), nerves (*e.g.*, neuromas, neuroblastomas), meninges (*e.g.*, Schwannomas and meningiomas) and eyes (*e.g.*, retinoblastomas); solid tumors arising from hematopoietic malignancies such as leukemias; and lymphomas including Hodgkin's and non-Hodgkin's lymphomas. The inventive methods may also be used to treat patients with prophylaxis of autoimmune diseases including rheumatoid, immune and degenerative arthritis. Other clinical conditions that may be treated using the inventive methods include ocular diseases such as diabetic retinopathy, corneal graft rejection, ocular neovascularization due to age-related macular generation, retinal vein occlusion, neovascular glaucoma; skin diseases such as psoriasis; blood vessel diseases such as hemagiomas and capillary proliferation within atherosclerotic plaques; myocardial angiogenesis; plaque neovascularization; atherosclerosis; and scleroderma.

[0155] In particular, when the bifunctional molecules of the invention are $\alpha_v\beta_3$-antagonists, they may be useful in the treatment of arthritic diseases, such as rheumatoid arthritis (C.M. Storgard *et al.*, J. Clin. Invest. 1999, 103: 47-54), in the suppression of proliferative diabetic retinopathy, which causes blindness in diabetic patients (M. Friedlander *et al.*, Proc. Natl. Acad. Sci. USA, 1996, 93: 9764-9769; H.-P. Hammes *et al.*, Nat. Med. 1996, 2: 529-533) and in the treatment of osteoclast-mediated osteoporosis (M.E. Duggan *et al.*, J. Med. Chem. 2000, 43: 3736-3745; W.H. Miller *et al.*, Bioorg. Med. Chem. Lett. 1999, 9: 1807-1812; V.W. Engleman *et al.*, J. Clin. Invest. 1997, 99: 2284-2292; M.W. Lark *et al.*, J. Bone Miner. Res. 2001, 16: 319-327).

[0156] The bifunctional molecules of the invention may also be used, singly or in combination with radiotherapy, surgery and/or other conventional chemotherapeutic treatments to prevent the formation of metastases in cancer patients.

V. Uses of the Targeted Contrast Imaging Agents

[0157] Another aspect of the present invention relates to methods for imaging angiogenesis in a system or a patient. Accordingly, the invention provides reagents and strategies to detect the presence of proliferating and/or migrating endothelial cells, of newly formed or growing blood vessels, of increased vascularity of an organ or a tissue of the body, or of metastases. More specifically, the invention provides targeted reagents that are detectable by imaging techniques and methods that allow the imaging of angiogenesis in *in vitro*, *in vivo*, and *ex vivo* systems as well as in patients. The methods provided herein are based on the use of inventive contrast imaging agents, which comprise at least one integrin-binding moiety associated with at least one imaging moiety that is detectable by imaging techniques.

[0158] More specifically, the present invention provides methods for imaging angiogenesis in a system comprising the step of contacting the system with an imaging effective amount of a contrast imaging agent of the invention, or a pharmaceutical composition thereof. The contacting is carried out under conditions that allow the contrast imaging agent to interact with the integrins present in the system so that the interaction results in the binding of the contrast imaging agent to the integrins. The presence of integrins bound to the contrast imaging agent is then detected using an imaging technique; and one or more images of at least part of the system are generated.

[0159] The contacting may be carried out by any suitable method known in the art. For example, the contacting may be carried out by incubation.

[0160] As already described above, the system may be any biological entity known in the art to be able to undergo angiogenesis, for example, the system may be a cell, a biological fluid, a biological tissue, or an animal. When the system is a cell, a biological fluid or a biological tissue, it may originate from a live patient (*e.g.*, it may be obtained by biopsy) or a deceased patient (*e.g.*, it may be obtained at autopsy). The patient may be a human or another mammal. In certain preferred embodiments, the cell, biological fluid, or biological tissue originates from a patient suspected to have a pathophysiological condition associated with angiogenesis. In other preferred embodiments, the cell, biological fluid, or biological tissue has been contacted (*in vitro* or *ex vivo*) with a potential therapeutic agent for the treatment of a pathophysiological condition associated with angiogenesis.

[0161] In a different aspect of the present invention, the method described above is used for identifying potential therapeutic agents. For example, images of at least part of a cell, biological fluid or biological tissue may be generated before and after contacting the cell, biological fluid or biological tissue with a potential therapeutic agent for the treatment of an angiogenic disorder. Comparison of the "before" and "after" images allows the determination of the effects of the agent on the system. The invention also includes the therapeutic agents useful for the treatment of pathophysiological conditions associated with angiogenesis identified by this method.

[0162] The present invention also provides methods for imaging angiogenesis in a patient. The methods comprise administering to the patient an imaging effective amount of a targeted contrast imaging agent of the invention, or a pharmaceutical composition thereof. The administration is carried out under conditions that allow the contrast imaging agent (1) to reach the area(s) of the patient's body that is/are affected by angiogenesis and (2) to interact with integrins present so that the interaction results in the binding of the contrast imaging agent to the integrins. After administration of the contrast imaging agent and after sufficient time has elapsed for the interaction to take place (for example, after between 30 minutes and 48 hours), the contrast imaging agent bound to integrins is detected using an imaging technique, and one or more images of at least part of the body of the patient are generated.

[0163] In one embodiment, these methods are used to localize angiogenesis (*i.e.*, to detect the presence of and localize proliferating and/or migrating endothelial cells, newly formed or growing blood vessels, increased vascularity of an organ or tissue of the body, or metastases) in a patient. By comparing the results obtained from a patient suspected of having a pathophysiological condition associated with angiogenesis and images obtained from studies of clinically healthy individuals, the diagnosis of the angiogenic disease can be confirmed.

[0164] Administration of the contrast imaging agent, or pharmaceutical composition thereof, can be carried out by any suitable method known in the art such as administration by oral and parenteral (*i.e.*, intravenous, intraarterial, intrathecal, intradermal, and intracavitory injections) methods, transdermal and topical methods.

[0165] In certain preferred embodiments, the methods provided herein to image angiogenesis in a system or a patient are carried out by using a contrast imaging agent

of the invention, wherein the metal-chelating moiety is complexed to a paramagnetic metal ion as described above. The imaging of angiogenesis is then performed by Magnetic Resonance Imaging (MRI), and magnetic resonance images are generated. Preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}).

[0166] In other preferred embodiments, the methods provided herein to image angiogenesis in a system or a patient are carried out by using a contrast imaging agent of the invention, wherein the metal-chelating moiety is complexed to a radionuclide as described above. The imaging of angiogenesis is then performed by Single Photon Emission Computed Tomography (SPECT), and SPECT images are generated. Preferably, the radionuclide is technetium-99m (^{99m}Tc).

[0167] In yet other preferred embodiments, the methods provided herein to image angiogenesis in a system or a patient are carried out by using a contrast imaging agent of the invention, wherein the metal-chelating moiety is complexed to a magnetically active nuclei as described above. The imaging of angiogenesis is then performed by Magnetic Resonance Spectroscopy (MRS), and magnetic resonance images are generated. Preferably, the magnetically active nuclei is the natural isotope carbon-13 (^{13}C) or fluorine-19 (^{19}Fl).

[0168] The inventive methods can be used to diagnose a pathophysiological condition associated with angiogenesis. The diagnosis can be achieved by examining and imaging parts or the whole body of the patient or by examining and imaging a biological system (such as one or more samples of biological fluid or biological tissue obtained from the patient). One or the other method, or a combination of both, will be selected depending on the nature of the clinical condition suspected to affect the patient. Comparison of the results obtained from the patient with data from studies of clinically healthy individuals will allow determination and confirmation of the diagnosis.

[0169] These methods can also be used to follow the progression of a pathophysiological condition associated with angiogenesis. For example, this can be achieved by repeating the method over a period of time in order to establish a time course for the presence, localization, distribution, and quantification of angiogenesis (*i.e.*, of proliferating and/or migrating endothelial cells, of newly formed or growing

blood vessels, of increased vascularity of an organ or tissue in the body, or of metastases) in a patient.

[0170] These methods can also be used to monitor the response of a patient to a treatment for a pathophysiological condition associated with angiogenesis. For example, an image of part of the body of the patient that undergoes angiogenesis (or an image of part of a cell, biological fluid, or biological tissue originating from the patient's body site affected by angiogenesis) is generated before and after submitting the patient to a treatment. Comparison of the "before" and "after" images allows to determine the effects of the treatment and therefore to monitor the response of the patient to that particular treatment.

[0171] The pathophysiological condition that may be diagnosed, or whose progression can be followed by the inventive methods are associated with angiogenesis and can affect any organ or tissue of the body. Organs such as heart, brain, lung, eye, gastro-intestinal tract, female and male genital tracts, skin, and the like, may be examined and imaged using the inventive methods provided herein.

[0172] Pathophysiological conditions that may be diagnosed, or whose progression may be followed by the inventive methods may be any of the diseases and disorders known in the art to be associated with an up-regulation of the angiogenic process leading to excessive blood vessel proliferation. For example, the inventive methods may be used to diagnose conditions including, but not limited to, primary and metastatic solid tumors and carcinomas of the breast; lung; gastro-intestinal tract; urinary tract; female genital tract; mal genital tract; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumors of the brain (*e.g.*, gliomas, gliobastomas), nerves (*e.g.*, neuromas, neuroblastomas), meninges (*e.g.*, Schwannomas and meningiomas) and eyes (*e.g.*, retinoblastomas); solid tumors arising from hematopoietic malignancies such as leukemias; lymphomas including Hodgkin's and non-Hodgkin's lymphomas. The inventive methods may also be used to treat patients with prophylaxis of autoimmune diseases including rheumatoid, immune and degenerative arthritis. Other clinical conditions include ocular diseases including diabetic retinopathy, corneal graft rejection, ocular neovascularization due to age-related macular generation, retinal vein occlusion, neovascular glaucoma; skin diseases including psoriasis; blood vessel diseases including hemagiomas and capillary proliferation within atherosclerotic plaques;

myocardial angiogenesis; plaque neovascularization; atherosclerosis; and scleroderma.

[0173] The contrast imaging agent of the invention may also be used to detect the presence, localize, and follow the progression of metastases in a patient with cancer.

VI. Uses of the Targeted Boron-Containing Compounds

[0174] Another aspect of the present invention relates to strategies for treating a patient with a tumor. Accordingly, the invention encompasses reagents and strategies that allow for the shrinkage of a tumor in a patient. In particular, the invention provides boron-containing compounds that target endothelial cells by having a high binding affinity and selectivity for integrins, and methods for using these compounds as therapeutics in Boron Neutron Capture Therapy.

[0175] More specifically, the present invention provides methods for treating a patient with a tumor comprising administering to the patient a BNCT effective amount of a boron-containing compound, or a pharmaceutical composition thereof. The administration is carried out under conditions that allow the boron-containing compound to reach the patient's body site(s) where the tumor is located and to selectively concentrate in the tumor by interacting with and binding to integrins. After administration of the boron-containing compound, the tumor and the compound are exposed to a neutron radiation beam of adequate energy. The neutron radiation is administered in a manner effective for the boron-containing compound in the tumor to be able to capture neutrons and emit α particles, and for a duration such that α particles are generated in an amount sufficient to shrink the tumor.

[0176] Administration of the boron-containing compounds, or pharmaceutical compositions thereof, may be performed by any suitable method known in the art, for example, by oral, transdermal and parenteral administrations (including intravenous, intramuscular, and subcutaneous injections). Typically, the boron-containing compounds are administered about 0.5 to about 30 days before exposure to radiation. Enough time must elapsed between administration and radiation for the concentration of the boron-containing compound in normal tissue and blood to be limited and preferably much less than the concentration in the tumor in order to minimize damage

to healthy tissues and blood vessels (H. Hatanaka *et al.*, in: "*Neutron Capture Therapy for Tumors*", Nishimura Co. 1986, pp. 1-16).

[0177] More than one BNCT agents may be administered to the same patient. For example, a boron-containing compound of the invention (*i.e.*, a molecule that targets endothelial cells by binding to integrins) may be administered in combination with a boron agent that has a high selectivity for malignant cells. The simultaneous irradiation of boron compounds with different localizations (*i.e.*, endothelial cells and malignant cells) allows for a dual mode of action and possibly, a more positive outcome of the treatment.

[0178] In BNCT, the neutron radiation beam used deliver thermal or epithermal neutrons (*i.e.*, neutrons of less than 0.5 eV to about 30-100 keV). Preferably, the neutrons delivered have an energy of less than about 0.5 eV to about 30 keV, and the neutron radiation beam has an incident gamma radiation dose rate within medically acceptable limits. Preferably, the neutron radiation beam has an energy distribution such that more than about 30 percent of the neutrons in the beam have energies which are less than about 0.5 eV and no more than 15 percent of the neutrons in the beam have energies which are greater than about 30 keV. For example, epithermal neutrons (1 eV < neutron energies < 10 keV) are optimal for the treatment of deep seated (2 cm deep) tumors as they provide a maximum neutron fluence at the tumor site with a minimum of damage to normal tissues.

[0179] The biological effect of the ^{10}B capture reaction, and thus its role in therapeutic action, does not result from the average effect on cells but rather from the combined effect on individual cells. Each cell is stochastically exposed to different amount of energy and will thus have a different chance of survival to another. Within this context, the calculation of an average dose is inadequate and it is necessary to undertake microdosimetric measurements and calculations. Thus, the treatment planning process involves incorporating the scans from an imaging technique (*e.g.*, MRI or CT); 3D reconstruction of defined anatomical regions of interest; 3D Monte Carlo radiation transport calculation of the neutron irradiation; calculation of the absorbed dose distribution; identification of optimal treatment position and time of irradiation required to deliver the prescribed doses; and evaluation of the dose distribution after the patient irradiation. Analytical and stochastic models for calculating microdosimetric parameters based on various scenarios of boron

subcellular distributions have been developed and are well-known to those skilled in the art (D. Gabel *et al.*, Radia. Res. 1987, 111: 14-25; R.A. Rydin *et al.*, Phys. Med. Biol. 1976, 21: 134-138; A.M. Kalend *et al.*, Int. J. Oncol. Biol. Phys. 1995, 31: 171-178).

[0180] The BNCT treatment of the invention may be administered in combination with other conventional therapies, such as surgery or chemotherapy. For example, the BNCT treatment may be administered after surgery to destroy the invasive parts of the tumor that are usually difficult to remove by surgery. The BNCT treatment may also be administered in combination with chemotherapeutic agents such as bleomycin, carboplatin, carmustine (BCNU), chlorambucil, cisplatin, cyclophosphamide, cytarabine, dacarbazine (DTIC), dactinomycin, daunorubicin, doxorubicin, etoposide, 5-fluorouracil, lomustine (CCNU), mechlorethamine, methotrexate, mitomycin, procarbazine, streptozocin, vinblastine, vincristine, or mixtures thereof.

[0181] The inventive methods are particularly useful for treating patient with brain tumors such as malignant gliomas of the brain, glioblastomas, astrocytomas, schwannomas, malignant meningiomas, oligodendrogliomas, ependymomas and the like, as well as metastatic tumors of the brain, which commonly arise from the lung, breast, prostate, skin, or gastro-intestinal tract. Malignant gliomas are brain tumors of the supportive tissue around the nerve cells. The prognostic of patients having received treatments (surgery, radiotherapy, and chemotherapy) for their malignant glioma is poor. Median survival from surgery is around one year and few patient live more than two years. Malignant gliomas are not clearly demarcated but are diffuse. The surrounding tissue is invaded by small paths of malignant cells along the nerve cells and blood vessels. About 90% of the tumor is in the main part and about 10% of the cells are in these invasive parts.

[0182] Other suitable tumors that may be treated by the inventive methods include malignant (*i.e.*, cancer) and non-malignant tumors of the skin, breast, uterus, prostate, bladder, pancreas, intestine, stomach, tyroid gland, ovary, kidney, lung, *etc.* The treatment is usually administered once but may be administered twice if necessary. The effectiveness of the treatment may be assessed by taking images of the site of the tumor during and immediately after the treatment. The long-term effects (recurrence of the tumor, metastasis, late toxicity from irradiation) may be monitored by biopsy, radiological methods and imaging techniques.

[0183] Some of the boron-containing compound of the invention may, in addition, be used as probes in a variety of diagnostic techniques, such as magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), and Single Photon Emission Computed Tomography (SPECT). Localization and quantification of the boron-containing compound within a patient (or within a sample of biological fluid or biological tissue obtained from the patient) may help in the planning of the BNCT treatment (for example in the determination of the most suitable time of irradiation, and in the microdosimetric measurements and calculations necessary for the determination of the irradiation dose to be administered).

VII. Formulation, Dosage and Administration

Bifunctional Therapeutic Molecules

[0184] The bifunctional therapeutic molecules described herein may be administered *per se* or in the form of a pharmaceutical composition. Accordingly, the present invention provides pharmaceutical compositions comprising an effective amount of at least one bifunctional molecule, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier. The specific formulation will depend upon the route of administration selected. The bifunctional molecules, or pharmaceutical compositions thereof, may be administered by any suitable method known in the art such as oral and parenteral administrations, including intravenous, intramuscular, intraperitoneal, and subcutaneous administrations, transdermal administrations, enteral administrations, and the like.

[0185] Pharmaceutical compositions for oral administration may be obtained by combining a bifunctional molecule of the invention with one or more pharmaceutically acceptable carriers or diluents. The use of such carriers allows the bifunctional molecules of the invention to be formulated, for example, as tablets, capsules, pills, dragees, liquids, gels, syrups, slurries, and suspensions. Pharmaceutically acceptable carriers and diluents for oral administration are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 1990), and include, but are not limited to, any and all solvents, dispersion media, antibacterial and antifungal agents, isotonic and absorption delaying agents. Such pharmaceutical compositions should contain at least 1% by weight of active compound. The percentage of the compositions may be varied and may conveniently be between

about 5 to about 80% of the weight of the unit. The amount of active compound in therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions according to the present invention are prepared so that an oral dosage unit form contains between about 0.5 μg and 2000 mg of active compound.

[0186] Oral formulations may optionally contain other conventional, non-toxic components such as fillers and binders (*e.g.*, sugars such as lactose, sucrose, mannitol, and sorbitol; and cellulose preparations such as starch, gelatin, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and polyvinylpyrrolidone); excipients (*e.g.*, dicalcium phosphate); disintegrating agents (*e.g.*, cross-linked polyvinyl pyrrolidone, agar, alginic acid, and sodium alginate); lubricants (*e.g.*, magnesium stearate); and flavoring agents (*e.g.*, peppermint, oil of wintergreen, and cherry flavoring). When the formulation forms a capsule, it may contain, in addition to materials listed above, liquid or semi-liquid vehicles (*e.g.*, fatty oils, liquid parrafin, and liquid polyethylene glycols). Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For example, tablets, pills, or capsules may be coated with shellac, sugar, or both. A syrup or elixir may contain the active compound, a sweetening agent such as sucrose, preservatives such as methyl and propylparabens, a dye, and flavoring such as cherry or orange flavor. Any material used in the preparation of oral pharmaceutical compositions should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0187] The bifunctional molecules of the invention may also be formulated for parenteral administration by injection (*e.g.*, by bolus injection or continuous infusion), and presented in unit dosage form (*e.g.*, in ampoules or in multi-dose containers). Dosage unit forms for injection are especially advantageous for ease of administration and uniformity of dosage. The term "***Dosage unit form***", as used herein, refers to a physically discrete unit suited as unitary dosage for the patient to be treated. Each unit contains a predetermined quantity of active material calculated to produce the desired therapeutic effect based on the characteristics of the active compound. For example, a unit dosage form contains the principal active ingredient in amounts ranging from 0.5 μg to about 2000 mg. Alternatively, amounts ranging from 200 ng/kg of body weight to above 10 mg/kg of body weight may be administered. The

amounts may be for individual active compounds or for the combined total of active compounds.

[0188] Parenteral compositions may be suspensions, emulsions, or aqueous and non-aqueous solutions of the active bifunctional molecule, and may optionally contain other auxiliaries such as suspending, stabilizing, and/or dispersing agents. Lipophilic solvents or vehicles (*e.g.*, fatty oils, synthetic fatty acid esters, and liposomes) can be used to prepare suspensions and emulsions. The viscosity of aqueous parenteral formulations may be increased by adding substances such as sodium carboxymethylcellulose, sorbitol, and dextran.

[0189] Alternatively, the bifunctional molecules of the invention may be formulated to allow a controlled delivery of the active ingredient. Control release compositions are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 1990) and may take the form of microcapsules, suppositories, or depot preparations. These pharmaceutical compositions may be obtained by incorporating or entrapping the active molecule(s) into particles of polymeric material (such as, for example, polyesters, polyamino acids, polyvinyl pyrrolidone, hydrogels, polylactic acid, ethylene vinylacetate, methylcellulose, hydroxymethylcellulose, and carboxy-methylcellulose) or in colloidal drug delivery systems (such as, for example, liposomes, microspheres, micro- or macro-emulsions, nanoparticles, and nanocapsules). Depot preparations may be administered by implantation or transcutaneous delivery, intramuscular injection, or through the use of a transdermal patch (see, for example, the devices described in U.S. Pat. Nos. 4,708,716 and 5,372,579).

[0190] The bifunctional therapeutic molecules of the invention, or pharmaceutical compositions thereof, may be administered singly, in combination with other reagents of the invention, and/or combined with other therapeutic agents, the nature of which will depend on the condition being treated. The ability to determine combinations of compounds suitable to treat particular disorders is well within the capabilities of trained physicians.

[0191] The bifunctional molecules of the invention, or pharmaceutical compositions thereof, can be administered therapeutically to treat a variety of pathophysiological conditions associated with angiogenesis, (*i.e.*, after the onset of the

disease) or prophylactically to prevent these pathophysiological conditions. In the case of cancer, the bifunctional molecules of the invention, or pharmaceutical compositions thereof, may be administered to prevent metastasis.

[0192] Administration of the bifunctional molecules of the invention, or pharmaceutical compositions thereof, will be in a dosage such that the amount delivered is effective for its intended purpose. The route of administration, formulation and dosage administered will be dependent upon the age, sex, weight and health condition of the patient; the particular pathophysiological condition to be treated; the extent of the disease; the potency, bioavailability, *in vivo* half-life and severity of the side effects of the bifunctional therapeutic molecule. These factors are readily determinable in the course of therapy. Alternatively or additionally, the dosage to be administered can be determined from studies using animal models for the particular condition being treated, and/or from animal or human data obtained for compounds which are known to exhibit similar pharmacological activities. The total dose required for each treatment may be administered by multiple dose or in a single dose. Adjusting the dose to achieve maximal efficacy based on these or other methods are well known in the art and within the capabilities of trained physicians.

[0193] Suitable patients with pathological conditions associated with angiogenesis can be identified by laboratory tests and medical history. In particular, the presence and location of proliferating endothelial cells, of newly formed or growing blood vessels, or of metastases can be determined by one of the inventive methods described herein, that use targeted contrast imaging agents and imaging techniques.

Contrast Imaging Agents

[0194] The present invention also provides pharmaceutical compositions comprising contrast imaging agents. More specifically, the pharmaceutical compositions of the invention comprise an imaging effective amount of at least one contrast imaging agent described above, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier. In certain preferred pharmaceutical compositions, the imaging moiety of the contrast imaging agent comprises at least one metal-chelating moiety complexed to a paramagnetic metal ion or to a radionuclide. Preferably, the paramagnetic metal ion is gadolinium III (Gd^{3+}); the radionuclide is technetium-99m (^{99m}Tc).

[0195] Administration of the contrast imaging agents, or pharmaceutical compositions thereof, may be carried out by any suitable method known in the art, such as those described in Remington's Pharmaceutical Sciences. Depending on the particular type of angiogenic disease suspected to affect the patient and the body site to be examined, the contrast imaging agent may be administered locally or systemically, and delivered orally (as solids, solutions, or suspensions) or by injection (for example, intravenously, intramuscularly, intraarterially, intrathecally (*i.e.*, via the spinal fluid), intradermally, or intracavitory).

[0196] For oral administration, the contrast imaging agents of the invention may be formulated as described above in the case of the bifunctional therapeutic molecules.

[0197] For administration by injection, pharmaceutical compositions of contrast imaging agents may be formulated as sterile aqueous or non-aqueous solutions or alternatively as sterile powders for the extemporaneous preparation of sterile injectable solutions. Such pharmaceutical compositions should be stable under the conditions of manufacture and storage, and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

[0198] Pharmaceutically acceptable carriers are solvents or dispersion media such as aqueous solutions (*e.g.*, Hank's solution, alcoholic/aqueous solutions, or saline solutions), and non-aqueous carriers (*e.g.*, propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate). Injectable pharmaceutical compositions may also contain parenteral vehicles (such as sodium chloride and Ringer's dextrose), and/or intravenous vehicles (such as fluid and nutrient replenishers); as well as other conventional, pharmaceutically acceptable, non-toxic excipients and additives including salts, buffers, and preservatives such as antibacterial and antifungal agents (*e.g.*, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal, and the like). Prolonged absorption of the injectable compositions can be brought about by adding agents that can delay absorption (*e.g.*, aluminum monostearate and gelatin). The pH and concentration of the various components can readily be determined by those skilled in the art.

[0199] Sterile injectable solutions are prepared by incorporating the active compound(s) in the required amount of the appropriate solvent with various of the

other ingredients enumerated above, followed by sterilization, for example, by filtration or irradiation. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying techniques.

[0200] Generally, the dosage of detectable contrast imaging agent will vary depending on considerations such as age, sex, and weight of the patient, as well as the particular pathophysiological condition suspected to affect the patient, the extent of the disease, and the area(s) of the body to be examined. Factors such as contraindications, concomitant therapies, and other variables are also to be taken into account to adjust the dosage of detectable contrast imaging agent to be administered. This can, however, be readily achieved by a trained physician. In general, a suitable daily dose of an inventive pharmaceutical composition corresponds to the lowest amount of contrast imaging agent that is sufficient to allow imaging of angiogenesis in the patient. To minimize this dose, it is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, and preferably proximal to the site to be examined. For example, intravenous administration is appropriate for imaging the urinary tract; intraspinal injection is better suited for imaging of the brain; while oral administration to an unfed patient is more appropriate for imaging of the gastrointestinal tract.

[0201] The radioactive contrast imaging agents of the invention are preferably administered in the range of about 0.1-10 mCuries/kg of body weight per day. The paramagnetic contrast imaging agents of the invention are preferably administered in the range of 0.02-1.3 mmoles/kg of body weight per day.

Targeted Boron-Containing Compounds

[0202] The present invention also provides the targeted boron-containing compounds described above in a pharmaceutically administrable form. More precisely, the invention provides pharmaceutical compositions comprising an effective amount of at least one boron-containing compound, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier. The specific formulation will mostly depend on the route of administration selected. The boron-containing compounds of the invention, or pharmaceutical compositions thereof, may be administered by any suitable method known in the art. Examples of

appropriate methods include, but are not limited to, transdermal, oral and parenteral (*i.e.*, subcutaneous, intravenous, or intramuscular injection) administrations. Depending on the intended mode of administration, the pharmaceutical composition may be in solid, semi-solid or liquid dosage form.

[0203] Pharmaceutical compositions for parenteral administration may be formulated as described in detail above for the targeted contrast imaging agents. Injectable suspensions of boron-containing compounds may also be prepared using liposomes as carriers according to methods well-known in the art (see, for example, Remington's Pharmaceutical Sciences, 1990 and U.S. Pat. No. 4,522,811). For example, a liposomal suspension may be formed by adding an aqueous solution of a boron-containing compound to a thin film of dried lipids, which may be obtained by dissolving lipids in an inorganic solvent and then evaporating said solvent. Suitable lipids for use in the present invention include low-density lipoproteins of any type, provided that they are high in cholesterol.

[0204] The parenteral pharmaceutical compositions of boron-containing compounds may be enclosed in ampoules, disposables syringes or multiple dose vials made of glass or plastic. The effective amount of boron-containing compound within a pharmaceutical composition will depend on factors such as absorption, distribution, inactivation, and excretion rates of the active ingredient. The boron-containing compounds are conveniently administered in any suitable unit dosage form, including but not limited to one containing about 0.5 μg to 3000 mg, preferably 5 to 500 mg of active ingredient per unit dosage form. A dosage form for intracarotid or intracerebral administration of approximately 5 to 500 mg is usually convenient.

[0205] Pharmaceutical compositions for oral administration may be formulated as described in detail above for the bifunctional therapeutic molecules. The amount of active ingredient in therapeutically useful oral compositions is such that suitable dosage will be obtained. Preferred compositions for oral administration are prepared so as to contain between about 0.5 μg and 3000 mg, preferably between 70 and 1400 mg of active ingredient per unit dosage form. An oral dosage of 50-1000 mg is usually convenient.

[0206] Pharmaceutical compositions of boron-containing compounds for topical administration include creams and ointments. The specific cream or ointment base to

be used in the formulation is one that is inert, stable, non-irritating and non-sensitizing and that will allow optimum drug delivery. As it is well-known in the art, ointments are semi-solid preparations that are typically based on petrolatum or other petroleum derivatives, while creams are viscous liquid or semi-solid emulsions, either oil-in-water or water-in-oil. Generally, cream bases are water-washable, and contain an oil phase (*e.g.*, comprised of petrolatum and a fatty alcohol such as cetyl or stearyl alcohol), an emulsifier (*e.g.*, a non-ionic, anionic, cationic or amphoteric surfactant), and an aqueous phase, which generally contains a humectant. For topical delivery, the boron-containing compounds of the invention may also be formulated to be administered through the skin or mucosal tissue using conventional transdermal drug delivery systems, as described above in detail for the bifunctional therapeutic molecules.

[0207] Boron-containing compounds may be administered alone or in combination (*i.e.*, together or alternatively) with other reagents of the invention or with other anti-cancer or chemotherapeutic compounds. Those include, but are not limited to, other boron compounds such as borocaptate sodium (BSH) and borono-phenalanine (BPA), or chemotherapeutic agents such as bleomycin, carboplatin, carmustine (BCNU), chlorambucil, cisplatin, cyclophosphamide, cytarabine, dacarbazine (DTIC), dactinomycin, daunorubicin, doxorubicin, etoposide, 5-fluorouracil, lomustine (CCNU), mechlorethamine, methotrexate, mitomycin, procarbazine, streptozocin, vinblastine, vincristine, or mixtures thereof.

[0208] When a boron-containing compound is to be administered together with one or more of the drugs listed above, these anti-cancer and chemotherapeutic agents may be included in the pharmaceutical composition provided that they do not affect the biological action of the boron-containing active ingredient. The pharmaceutical compositions may also contain other active materials such as, for example, antibiotics, anti-fungals, anti-inflammatories, and anti-viral compounds. Such active materials will depend upon the specific disease to be treated.

[0209] Administration of the boron-containing compounds may be performed in a single dose, continuously (*e.g.*, intravenous drip), or in multiple doses over a certain period of time. The dosage will vary depending on several factors such as the particular boron-containing compound to be administered and its characteristics (*e.g.*, the potency, bioavailability, *in vivo* half-life and severity of the side effects), the

formulation and route of administration selected, the tumor type, the extent of the disease, as well as the age, sex, weight and general health condition of the patient to be treated. Selecting the dosage regimen and adjusting the dose to achieve maximal efficacy based on these or other methods are well within the capabilities of trained physicians.

[0210] Generally, however, dosage will be in the range of 0.01 and between approximately 500 and 1000 mg/kg of body weight per day; preferably in the range of about 0.5 and between approximately 20 to 100 mg/kg of body weight per day. When the boron-containing compound is co-administered with one or more other active materials, a lower dosage may be administered.

[0211] The effectiveness of the treatment may be monitored by different methods known in the art such as tumor biopsy, or radiological methods. The anti-angiogenic activity of the treatment may also be monitored using one of the methods described herein that involve the use of targeted contrast imaging agents and imaging techniques.

Examples

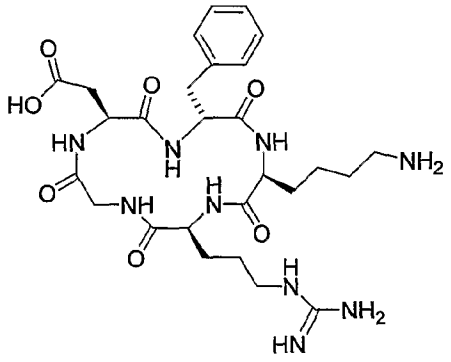
[0212] The following examples describe some of the preferred modes of making and practicing the present invention. However, it should be understood that these examples are for illustrative purposes only and are not meant to limit the scope of the invention. Furthermore, unless the description in an Example is presented in the past tense, the text, like the rest of the specification is not intended to suggest that experiments were actually performed or data were actually obtained.

Example 1: Synthesis of an Integrin-binding, Metal-chelating Therapeutic Molecule

[0213] The synthesis of a bifunctional molecule comprising one metal-chelating moiety directly covalently linked to two identical integrin-binding moieties is described herein. The metal-chelating moiety is diethylenediaminetetraacetic acid, DTPA, a metal chelator well-known in the art. The integrin-binding moiety is a cyclic derivative of RGDfV, a RGD-containing peptide (R. Haubner *et al.*, J. Am. Chem. Soc. 1996, 118: 7461-7472) that has been reported to be one of the most

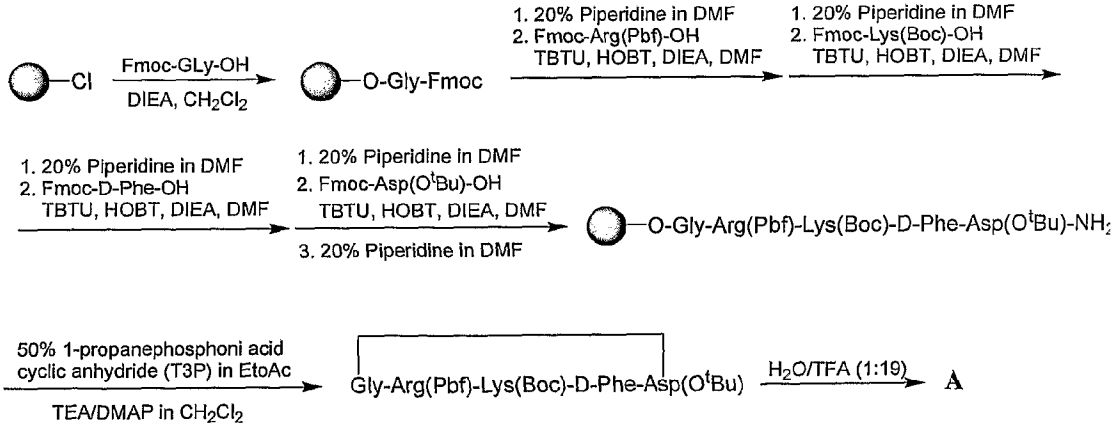
selective for the integrin $\alpha_v\beta_3$ (M.A. Dechantsreiter *et al.*, J. Med. Chem. 1999, 42: 3033-3040).

[0214] The first part of the synthesis provides the *cyclo*(-RGDfK-) peptide, or compound **A**, the chemical structure of which is depicted below.



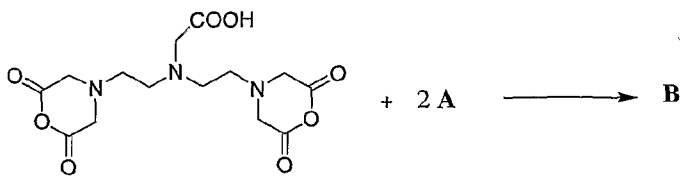
A

The reaction, shown in the following scheme, is carried out as previously described (in X. Dai *et al.*, Tetrahedron Lett. 2000, 41: 6295-6298), by solid-phase synthesis using a 2-chlorotrityl chloride resin. The reaction gives (up to several grams of) compound **A** with an overall yield of about 79%.

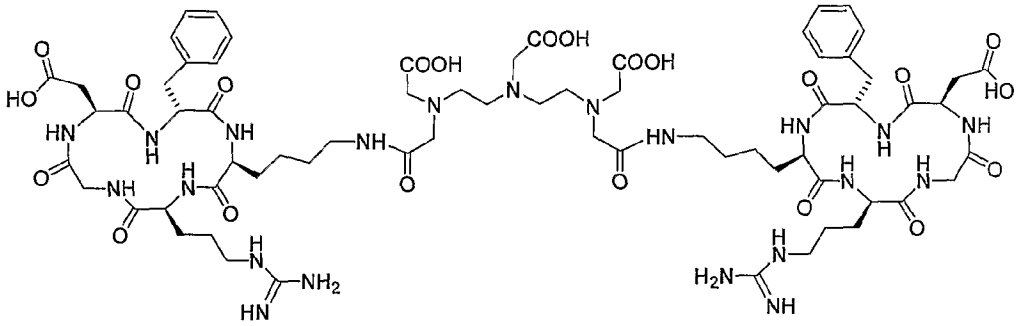


[0215] The second part of the synthesis, shown in the scheme below, leads to the desired bifunctional molecule. It involves the formation of amide bonds between the metal-chelating moiety and the integrin-binding moieties. The reaction is carried out according to a procedure adapted from previously published synthetic methods (W.E.

Klunk *et al.*, Life Sci. 2001, 69: 1471-1484; D. Shi *et al.*, J. Med. Chem. 1996, 39: 3375-3384; and M.S. Konings *et al.*, Inorg. Chem. 1990, 29: 1488-1491).



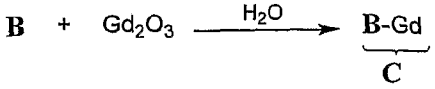
where **B** is:



[0216] More specifically, DTPA-bis(anhydride) (2.5 g, 7.0 mmol) is added in portions over 30 minutes to an ice-cold stirred solution of compound **A** (8.85 g, 7.8 mmol) in ethanol. After addition of water (150 mL), the resulting reaction mixture is stirred for another 12 hours at ambient temperature. The solution obtained by concentrating the reaction mixture under reduced pressure and adding water (500 mL) is adjusted to pH 2.5 with concentrated HCl to induce the formation of crystals. After collection, the crystals are recrystallized from ethanol to give compound **B** (71%).

Example 2: Synthesis of an MRI Contrast Imaging Agent

[0217] A contrast imaging agent detectable by Magnetic Resonance Imaging (MRI) can be prepared from the bifunctional molecule described in Example 1. As shown below, synthesis of the MRI contrast imaging agent **C**, involves insertion of gadolinium III (Gd^{3+}). This reaction is carried out according to a method previously reported (M.S. Konings *et al.*, Inorg. Chem. 1990, 29: 1488-1491).



[0218] More specifically, a mixture of compound **B** (12.1 g, 25.0 mmol) and gadolinium oxide, Gd_2O_3 (4.53 g, 12.4 mmol), in water (30 mL) is refluxed for 5 hours. Colorless crystals of the gadolinium complex, compound **C**, are quantitatively formed by adjusting the pH of the solution to 6.5 with 1 M NaOH.

Example 3: Synthesis of a SPECT Contrast Imaging Agent

[0219] A contrast imaging agent detectable by Single Photon Emission Computed Tomography (SPECT), can be prepared from the bifunctional molecule described in Example 1. The reaction, which is shown below, is carried out by inserting technetium-99m using the stannous reduction at pH 6.5 procedure described in U.S. Pat. No. 4,434,151. This reaction yields the technetium complex, compound **D**, quantitatively.

$$\mathbf{B} + {}^{99m}TcO_4^- \xrightarrow{SNCl_2 \cdot 2H_2O} \underbrace{\mathbf{B}\text{-}{}^{99m}Tc}_{\mathbf{D}}$$

[0220] More specifically, compound **B** (8.25 g, 0.17 mmol) is dissolved in 1.0 mL of ethanol and 1.0 M sodium acetate at pH 5.5, and 1.0 mL generator eluant of $^{99m}TcO_4^-$ (5-50 mCi) in saline is added to the reaction mixture. Addition of 0.2 mL of a stannous solution, prepared by dissolving 2.0 mg of $SnCl_2 \cdot H_2O$ per mL of ethanol, produced compound **D**. After 15-30 minutes, the labeling efficiency can be determined by electrophoresis.

Claims

What is claimed is:

1. A bifunctional molecule comprising at least one metal-chelating moiety associated with at least one integrin-inhibiting moiety.

2. The bifunctional molecule of claim 1, wherein the metal-chelating moiety binds with high affinity copper II (Cu^{2+}) or iron III (Fe^{3+}), or both.

3. The bifunctional molecule of claim 1, wherein the metal-chelating moiety binds with high affinity and specificity copper II (Cu^{2+}).

4. The bifunctional molecule of claim 1, wherein the integrin-inhibiting moiety has a high affinity and specificity for integrins.

5. The bifunctional molecule of claim 1, wherein the integrin-inhibiting moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$, or the integrin $\alpha_v\beta_5$, or both.

6. The bifunctional molecule of claim 1, wherein the integrin-inhibiting moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$.

7. A contrast imaging agent comprising at least one imaging moiety associated with at least one integrin-binding moiety.

8. The contrast imaging agent of claim 7, wherein the integrin-binding moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$, or the integrin $\alpha_v\beta_5$, or both.

9. The contrast imaging agent of claim 7, wherein the integrin-binding moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$.

10. The contrast imaging agent of claim 7, wherein the imaging moiety comprises at least one metal-chelating moiety complexed to a metal entity.

11. The contrast imaging agent of claim 10, wherein the metal entity is a paramagnetic metal ion.

12. The contrast imaging agent of claim 10, wherein the metal entity is a paramagnetic metal ion selected from the group consisting of gadolinium III (Gd^{3+}), chromium III (Cr^{3+}), dysprosium III (Dy^{3+}), iron III (Fe^{3+}), manganese II (Mn^{2+}), and ytterbium III (Yb^{3+}).

13. The contrast imaging agent of claim 10, wherein the metal entity is gadolinium III (Gd^{3+}).

14. The contrast imaging agent of claim 10, wherein the metal entity is a radionuclide.

15. The contrast agent of claim 10, wherein the metal entity is a radionuclide selected from the group consisting of technetium-99m (^{99m}Tc), gallium-67 (^{67}Ga), yttrium-91 (^{90}Y), indium-111 (^{111}In), rhenium-186 (^{186}Re), and thallium-201 (^{201}Tl).

16. The contrast imaging agent of claim 10, wherein the metal entity is technetium-99m (^{99m}Tc).

17. A boron-containing compound comprising at least one integrin-binding moiety associated with at least one boron-containing moiety.

18. A boron-containing compound comprising at least one metal-chelating moiety associated with at least one integrin-binding moiety labeled with Boron-10.

19. A boron-containing compound comprising at least one imaging moiety associated with at least one integrin-binding moiety labeled with Boron-10.

20. The boron-containing compound of claim 18 or 19, wherein the integrin-binding moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$, or the integrin $\alpha_v\beta_5$, or both.

21. The boron-containing compound of claim 18 or 19, wherein the integrin-inhibiting moiety has a high affinity and specificity for the integrin $\alpha_v\beta_3$.

22. A pharmaceutical composition comprising an effective amount of at least one bifunctional molecule of claim 1, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier.

23. A pharmaceutical composition comprising an imaging effective amount of at least one contrast imaging agent of claim 7, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier.

24. A pharmaceutical composition comprising an effective amount of at least one boron-containing compound of claim 17, 18 or 19, or a physiologically tolerable salt thereof, and at least one pharmaceutically acceptable carrier.

25. A method for preventing or inhibiting angiogenesis in a system, comprising contacting the system with a bifunctional molecule of claim 1, or a pharmaceutical composition thereof.

26. The method of claim 25, wherein said method prevents or inhibits angiogenesis by inhibiting the biological activity of integrins.

27. The method of claim 26, wherein said method inhibits the biological activity of integrins by precluding, reversing, disrupting, or otherwise interfering with the interaction of integrins with their endogenous ligands.

28. The method of claim 25, wherein said method prevents or inhibits angiogenesis by inhibiting the biological activity of angiogenic stimulators that require the presence of copper II as co-factor.

29. The method of claim 28, wherein the method inhibits the biological activity of angiogenic stimulators that require the presence of copper II as co-factor, by precluding, reversing, disrupting or otherwise interfering with the interaction between said angiogenic stimulators and copper II.

30. The method of claim 25, wherein the contacting is carried out by *in vitro* or *ex vivo* incubation, and wherein the system is selected from the group consisting of a cell, a biological fluid, and a biological tissue.

31. The method of claim 30, wherein the cell, biological fluid, or biological tissue originates from a patient suspected to have a pathophysiological condition associated with angiogenesis.

32. A method for treating a patient with a pathophysiological condition associated with angiogenesis, comprising administering to the patient an effective amount of a bifunctional molecule of claim 1, or a pharmaceutical composition thereof.

33. The method of claim 32, wherein the administration is carried out by a method selected from the group consisting of oral and parenteral administrations, including intravenous, intramuscular, subcutaneous, and transdermal administrations, and transdermal and topical administration.

34. The method of claim 31 or 32, wherein the pathophysiological condition is selected from the group consisting of cancer, psoriasis, atherosclerosis, restenosis, rheumatoid arthritis, and ocular neovascularization

35. A method for imaging angiogenesis in a system comprising:

contacting the system with an imaging effective amount of a contrast imaging agent of claim 7, or a pharmaceutical composition thereof, under conditions that allow the contrast imaging agent to interact with integrins present so that the interaction results in the binding of the contrast imaging agent to the integrins;

detecting the contrast imaging agent bound to integrins present in the system, using an imaging technique; and

generating one ore more images of at least part of the system.

36. The method of claim 35, wherein the contacting is carried out by *in vitro* or *ex vivo* incubation, and wherein the system is selected from the group consisting of a cell, a biological fluid, and a biological tissue.

37. The method of claim 35, wherein the cell, biological fluid, or biological tissue originates from a patient suspected of having a pathophysiological condition associated with angiogenesis.

38. The method of claim 35, wherein the cell, biological fluid, or biological tissue originates from a patient receiving a treatment for a pathophysiological condition associated with angiogenesis.

39. The method of claim 35, wherein the cell, biological fluid, or biological tissue has been contacted with a potential therapeutic agent for the treatment of a pathophysiological condition associated with angiogenesis.

40. A method for imaging angiogenesis in a patient comprising:

administering to the patient an imaging effective amount of a contrast imaging agent of claim 7, or a pharmaceutical composition thereof, under conditions that allow the contrast imaging agent to interact with integrins present so that the interaction results in binding of the contrast imaging agent to the integrins;

detecting the contrast imaging agent bound to integrins present in the patient, using an imaging technique; and

generating one ore more images of at least part of the body of the patient.

41. The method of claim 40, wherein the administration is carried out by a method selected from the group consisting of oral and parenteral administrations, including intravenous, intraarterial, intrathecal, intradermal, and intracavitory administrations, and transdermal and topical administrations.

42. The method of claim 35 and 40, wherein the imaging moiety in the contrast imaging agent comprises at least one metal-chelating moiety complexed to a paramagnetic metal ion; the detection is carried out by Magnetic Resonance Imaging (MRI); and magnetic resonance images are generated.

43. The method of claim 42, wherein the paramagnetic metal ion is selected from the group consisting of gadolinium III (Gd^{3+}), chromium III (Cr^{3+}), dysprosium III (Dy^{3+}), iron III (Fe^{3+}), manganese II (Mn^{2+}), and ytterbium III (Yb^{3+}).

44. The method of claim 42, wherein the paramagnetic metal ion is gadolinium III (Gd^{3+}).

45. The method of claim 35 and 40, wherein the imaging moiety in the contrast imaging agent comprises at least one metal-chelating moiety complexed to a radionuclide; the detection is carried out by Single Photon Emission Computed Tomography (SPECT); and SPECT images are generated.

46. The method of claim 45, wherein the radionuclide is selected from the group consisting of technetium-99m (^{99m}Tc), gallium-67 (^{67}Ga), yttrium-91 (^{90}Y), indium-111 (^{111}In), rhenium-186 (^{186}Re), and thallium-201 (^{201}Tl).

47. The method of claim 45, wherein the radionuclide is technetium-99m (^{99m}Tc).

48. The method of claim 35 and 40, wherein the integrin-binding moiety in the contrast imaging agent is labeled with a magnetically active nuclei; the detection is carried out by Magnetic Resonance Spectroscopy (MRS); and MR images are generated.

49. The method of claim 48, wherein the magnetically active nuclei is carbon-13 (^{13}C) or fluorine-19 (^{19}F).

50. The method of claim 35, wherein said method is used to identify potential therapeutic agents for the treatment of a pathophysiological condition associated with angiogenesis.

51. The method of claim 35, wherein said method is used to diagnose a pathophysiological condition associated with angiogenesis.

52. The method of claim 35, wherein said method is used to follow the progression of a pathophysiological condition associated with angiogenesis.

53. The method of claim 35, wherein said method is used to monitor the response of a patient to a treatment for a pathophysiological condition associated with angiogenesis.

54. The method of claim 40, wherein said method is used to localize angiogenesis in a patient.

55. The method of claim 40, wherein said method is used to diagnose a pathophysiological condition associated with angiogenesis.

56. The method of claim 40, wherein said method is used to follow the progression of a pathophysiological condition associated with angiogenesis.

57. The method of claim 40, wherein said method is used to monitor the response of a patient to a treatment for a pathophysiological condition associated with angiogenesis.

58. The method of claim 51, 52, 53, 54, 55, 56 or 57, wherein the pathophysiological condition is selected from the group consisting of cancer, psoriasis, atherosclerosis, restenosis, rheumatoid arthritis, and ocular neovascularization

59. A method for treating a patient with a tumor comprising:

administering to the patient a BNCT effective amount of a boron-containing compound of claim 17, 18 or 19, or a pharmaceutical composition thereof, under conditions that allow the boron-containing compound to reach the part of the patient where the tumor is located and to selectively concentrate in the tumor by binding to integrins; and

exposing the tumor and the boron-containing compound bound to integrins to a neutron radiation beam of an energy such that said compound emits alpha particles in an amount sufficient to shrink said tumor.

60. The method of claim 59, wherein said neutron radiation beam energy ranges from about 0.5 eV to about 30 keV.

61. The method of claim 59, wherein said neutron radiation beam has an energy distribution such that no more than about 30 percent of the neutrons in said beam have energies which are less than about 0.5 eV and no more than 15 percent of the neutrons in said beam have energies which are greater than about 30 keV and has an incident gamma radiation dose rate within medically acceptable limits.

62. The method of claim 59, wherein said tumor is a brain tumor.

63. The method of claim 59, wherein said tumor is a malignant glioma of the brain.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/002693

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K49/00 A61K51/08 A61K49/14 A61K41/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/40947 A (ESHIMA DENNIS ; POLLAK ALFRED (CA); THORNBACK JOHN (CA); FAUCONNIER TH) 19 August 1999 (1999-08-19) claims 1,7,8,17,22,32; examples 2,8 page 19, lines 1-4 page 20, line 13 page 15, lines 15-17 -/--	1,4-15, 22,23, 25-44, 51,54, 55,58

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 June 2004	06/07/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Allnutt, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/32292 A (TRUSTESS OF THE LELAND STANFOR) 25 April 2002 (2002-04-25) page 8, line 19; claims 1,2,7,9,10,15,19 page 11, lines 13-15; examples 2,3 page 6, lines 11-17	1,4-12, 14-16, 22,23, 25-29, 35-48, 50-58
X	HAUBNER R ET AL: "Radiolabeled alpha(v)beta3 integrin antagonists: a new class of tracers for tumor targeting." JOURNAL OF NUCLEAR MEDICINE : OFFICIAL PUBLICATION, SOCIETY OF NUCLEAR MEDICINE. JUN 1999, vol. 40, no. 6, June 1999 (1999-06), pages 1061-1071, XP009032232 ISSN: 0161-5505 cited in the application page 1062, column 1, lines 3-17 - column 2, lines 11-19 page 1065, column 1, lines 53-55 see conclusion page 1066, column 2, lines 5-11	1,4-10, 14-16, 22,23, 25-29, 32-35, 37-41, 45-49, 51,52, 54-56,58
X	WO 02/087498 A (UNIV TEXAS) 7 November 2002 (2002-11-07) claims 1,4,21,23,31,45 page 15, line 32 page 16, lines 19-23 page 17, lines 7-13	1-10, 14-16, 22,23, 32-35, 37-41, 45-47, 51,52, 54-56,58
X	WO 03/006491 A (INDREVOLL BAARD ; SOLBAKKEN MAGNE (NO); AMERSHAM HEALTH AS (NO); CUTHB) 23 January 2003 (2003-01-23) claims 14,15,24,26-28 page 6, lines 15-19 page 22, line 8 - page 23, line 12 page 27, lines 8-11	1,4-11, 14-16, 22,23, 32-35, 37-42, 48,49, 51-58

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/002693

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HALLAHAN D E ET AL: "Targeting drug delivery to radiation-induced neoantigens in tumor microvasculature" JOURNAL OF CONTROLLED RELEASE, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, NL, vol. 74, no. 1-3, 6 July 2001 (2001-07-06), pages 183-191, XP004297523 ISSN: 0168-3659 abstract page 188, column 2 - page 189, column 1, paragraph 1	1,4-10, 14-16, 22,23
X	WO 99/58162 A (DU PONT PHARM CO) 18 November 1999 (1999-11-18) page 8, lines 5-7; claims 11,12,19,32-37	1,4-12, 14-16, 22,23, 32-35, 37-48, 51-58
X	WO 00/64480 A (IMP CANCER RES TECHNOLOGY LTD ; BICKNELL ROY (GB); ZHANG HUA TANG (GB)) 2 November 2000 (2000-11-02) page 20, lines 19-27; claims 1,5,13,17,28,29	1,4-16, 22,23, 25-29, 31-35, 37-44, 48,49, 51-58
A	BREWER G J ET AL: "TREATMENT OF METASTATIC CANCER WITH TETRATHIOMOLYBDATE, AN ANTICOPPER, ANTIANGIOGENIC AGENT: PHASE I STUDY" January 2000 (2000-01), CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, PAGE(S) 1-10 , XP000906939 ISSN: 1078-0432 cited in the application abstract	24-31
A	US 5 567 408 A (ZAMORA PAUL O) 22 October 1996 (1996-10-22) cited in the application the whole document	1-16

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 25-29 and 59-63 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
Although claims 35,37-58 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.1

Claims Nos.: 25-29,32-35,37-63

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

International application No:
PCT/US2004/002693

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 25-29,32-35,37-63
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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